UNIVERSIDAD AUTÓNOMA DE MADRID

ESCUELA DE DOCTORADO

Validation of coffee by-products as food ingredients for a sustainable nutrition and health

Validación de subproductos de café como ingredientes alimentarios para una nutrición y salud sostenible

Amaia Iriondo De Hond

Madrid, October 2019

CIAL
INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

María Dolores del Castillo, Dra. en Ciencia y Tecnología de los Alimentos e Investigadora Científica del Instituto de Investigación en Ciencias de la Alimentación (CIAL) del Consejo Superior de Investigaciones Científicas (CSIC),

Informa:

Que el presente trabajo titulado "**Validation of coffee by-products as food ingredients for a sustainable nutrition and health"** (Validación de subproductos de café como ingredientes alimentarios para una nutrición y salud sostenible) constituye la memoria que presenta Amaia Iriondo De Hond, Bioquímica por la Universidad Autónoma de Madrid (UAM), para optar al GRADO DE DOCTORA. La presente memoria ha sido realizada bajo su dirección en el Departamento de Bioactividad y Análisis de Alimentos del CIAL (UAM-CSIC) y reúne las condiciones necesarias para su presentación y defensa.

Y para que conste a los efectos oportunos, firma el presente certificado,

Fdo.: Dra. M.ª Dolores del Castillo Bilbao

En Madrid, Octubre de 2019

“Change the world. Start with coffee”

Anonymous

ACKNOWLEDGEMENTS

"Work hard at being the best version of yourself"

Isadora Duncan

I would like to give thanks to the people that have been part of my PhD journey and have assisted me at every point to reach this goal. As Nelson Mandela said, "*If you talk to a man in a language he understands, that goes to his head. If you talk to him in his own language, that goes to his heart.*" Therefore, I will continue with these words of gratitude in Spanish.

Me gustaría empezar dando las gracias a mi directora de tesis, Dra. Mª Dolores del Castillo, por haberme dado la oportunidad de realizar esta tesis doctoral. Loli, gracias por tu apoyo, entusiasmo y por la confianza que has puesto en mi desde el primer día. Gracias a ti he aprendido que es posible combinar la Bioquímica con las Ciencias de la Alimentación, que el buen trabajo y el esfuerzo tienen su recompensa y a mantener la calma en los momentos difíciles. Has sido mi directora, tutora, jefa, mentora, compañera y amiga. Siempre recordaré felizmente las aventuras que vivimos durante nuestra estancia en Boston, entre las cuales me di cuenta de que el verdadero reto en la vida es comer espaguetis con tomate llevando un jersey blanco, que los *leggins* encogen en la secadora, que si te descuidas y dejas un plátano en el balcón se congela, y… ¡que la vida sigue!

A continuación, quiero agradecer al antiguo Ministerio de Economía y Competitividad (MINECO) por concederme la ayuda predoctoral y al Instituto de Investigación en Ciencias de la Alimentación (CIAL, UAM-CSIC) por haberme permitido llevar a cabo las investigaciones de mi tesis doctoral en sus instalaciones.

Una de las ventajas de pertenecer a la pequeña familia de Biociencia de los Alimentos, ha sido la posibilidad de colaborar, realizar estudios y conocer a grandes investigadores de otras instituciones. Gracias a las Dras. Paloma Morales y Ana I. Haza de la Facultad de Veterinaria de la Universidad Complutense de Madrid (UCM) por haberme acogido en su grupo y haberme enseñado la apasionante técnica Comet para realizar los estudios de genotoxicidad. De esta misma facultad me gustaría agradecer a los Dres. Manuel Ignacio San Andrés y Sebastián Sánchez-Fortún por sus enseñanzas y apoyo con los estudios en animales. Nunca olvidaré el gran equipo que formamos el último día del

estudio de dosis repetidas. Estos estudios *in vivo* no habrían sido posibles sin la ayuda de Elena y Fernando, responsables del animalario de Centro de Biología Molecular Severo Ochoa (CBMSO), que nos ayudaron y dieron todas las facilidades para llevar a cabo estos estudios.

Quisiera expresar mi agradecimiento al Dr. Jin-Rong Zhou del Beth Israel Deaconess Medical Center (BIDMC) por habernos recibido con los brazos abiertos en su grupo y hacer de nuestra estancia en Boston una experiencia de lo más enriquecedora. También me gustaría dar las gracias a las Dras. Raquel Ábalo y Gema Vera y a todo el grupo de Farmacología de la Universidad Rey Juan Carlos (URJC) por haberme enseñado los diferentes ensayos de motilidad intestinal, ¡nunca pensé que haría radiografías a unas ratitas!

Me gustaría dar las gracias a todos los compañeros del CIAL que me han acompañado durante estos años. A Macarena, Javier, Beatriz, Jesús, Julia y Juan Antonio, por recibirme con una sonrisa y ayudarme con las gestiones y dudas administrativas que os suelo llevar a la planta baja. Gracias a Elena y Juan Antonio de la BAT por su apoyo y su alegría a tempranas horas de la mañana por los laboratorios P2. También a otro madrugador en potencia, el Dr. Jose Manuel Silván, que hace las mañanas en cultivos más divertidas y alegres. A continuación, les doy las gracias a mis vecinos de Carbohidratos, Paloma, Agustina, Pablo y Álvaro que me han acompañado en los últimos días y especialmente a Gorka por su alegría de vivir y esos buenos momentos en el laboratorio. Y por supuesto, a Nerea, por estar siempre ahí y compartir esta aventura del doctorado de la mano. Gracias por alegrarme estos últimos meses, por apoyarme y por darme las predicciones de sexo y fecha de nacimiento de mi sobrin@, ¡eres lo más!

Por supuesto, al resto de la familia de Biociencia, Nuri, Bea, Moni, Miguel, Adri, Tere, Bruno y a los mejores italianos que podrían habernos visitado Alessandro y Marco, gracias por vuestro apoyo y por entender y soportar mi trastorno con el orden. Un mundo ordenado es un mundo mejor. Gracias también a mis bioquímicas, Alicia, Inés y Marta que, con dos quedadas anuales, nos ponemos al día y nos entendemos perfectamente.

Me gustaría agradecer especialmente a mis grandes amigos Carmen, Rafa, Elena, Alberto y Álvaro, por apoyarme durante todos estos años, sois los mejores. No os dais cuenta de lo mucho que me ha ayudado quedar con vosotros este verano. Y no me podía olvidar de Conchita, Bea y Clara, mi familia de Auladanza. Gracias por hacer de la danza un pilar esencial en mi vida que sin vosotras no tendría sentido. Gracias a todos los que me habéis preguntado "¿y sigues trabajando en eso del café?" y que después me habéis

escuchando pacientemente como os explicaba que en realidad yo trabajaba con los subproductos, y que el mundo del café es verdaderamente apasionante.

Por último, me gustaría destacar especialmente el apoyo que me ha dado mi familia. Gracias Kiwi, que sobre todo durante los dos primeros años de tesis me has acompañado y mostrado tu apoyo durmiendo al lado del ordenador, trasmitiéndome esa paz que te caracteriza. Gracias a mis padres, que me han ayudado incondicionalmente. Mami, Aita, sois el ejemplo de fortaleza, esfuerzo y superación. Gracias por estar ahí en todo momento, os admiro y os quiero un montón. Una de las mejores cosas de esta tesis ha sido poder trabajar junto a mi hermana mayor, Maite. Ha sido maravilloso poder hacer la tesis juntas. Me ha encantado venir contigo a las 7 de la mañana al laboratorio, disfrutar del buen café, intentar ponernos a escribir y no poder parar de hablar (o de comer, jaja) y escribir trabajos que sean Iriondo-DeHond, Iriondo-DeHond. Solo tú eres capaz de hacer que subir al cercanías cuando dentro hacía 40 grados sea tan divertido. Me siento muy afortunada de tenerte como hermana y amiga. No podría tener mejor ejemplo que seguir en la vida. Igual de afortunada me siento de tener a Dani como cuñado. Otro ejemplo de ser trabajador y, sobre todo, buena persona.

El mayor agradecimiento se lo quiero dar a Andrés, que en estos 10 años me ha hecho feliz, y me ha cuidado y amado. Muchísimas gracias por apoyarme en este reto día a día, y aguantar a mi verdadero yo. Esta ha sido otra aventura más en nuestra historia junto con todas las que nos quedan por vivir. Por eso, le diremos a Eduardo que nos lleve a Cantocochino, que no queremos ver el mar.

Y ahora…

“Volare oh, oh
Cantare oh, oh
Nel blu dipinto di blu
Felice di stare lassù”

GRANTS AND FUNDING

The present study has been funded by the following project:

1. Producción y consumo sostenibles del café: validación de subproductos como ingredientes alimentarios (SUSCOFFEE, AGL2014-57239-R) funded by the Ministry of Economy and Competitiveness.

Amaia Iriondo De Hond received financial support with a PhD scholarship funded by the Ministry of Economy and Competitiveness of Spain (BES-2015-072191).

LIST OF PUBLICATIONS

Research from this PhD Thesis resulted in the following publications:

Research articles:

- **Iriondo-DeHond, A**., Ríos, M.B., Herrera, T., Rodríguez-Bertos, A., Nuñez, F., San Andres, M.I., Sanchez-Fortun, S., del Castillo, M.D. (2019). Coffee silverskin extract: Nutritional value, safety and effect on key biological functions. Submitted to *Nutrients.*
- **Iriondo-DeHond, A**., Cornejo, F.S., Fernandez-Gomez, B., Vera, G., Guisantes-Batan, E., Alonso, S.G., San Andres, M.I., Sanchez-Fortun, S., Lopez-Gomez, L., Uranga J.A., Abalo, R., del Castillo, M.D. (2019). Bioaccessibility, Metabolism, and Excretion of Lipids Composing Spent Coffee Grounds. *Nutrients, 11*(6), 1411. https://doi.org/10.3390/nu11061411.
- **Iriondo-DeHond A**., Ramirez B., Velazquez-Escobar F.V., del Castillo M.D. (2019) Antioxidant properties of high molecular weight compounds from coffee roasting and brewing byproducts. *Bioactive Compounds in Health and Disease, 2*(3):48-63. https://doi.org/10.31989/bchd.v2i3.588.
- Tores de la Cruz, S., **Iriondo-DeHond, A**., Herrera, T., Lopez-Tofiño, Y., Galvez-Robleño, C., Prodanov, M., Velazquez-Escobar, F., Abalo, R., del Castillo, M.D. (2019). An assessment of the bioactivity of coffee silverskin melanoidins. *Foods,* 1–21. https://doi.org/10.3390/foods8020068.
- **Iriondo-DeHond, A**., Aparicio García, N., Fernandez-Gomez, B., Guisantes-Batan, E., Velazquez Escobar, F., Blanch, G.P., San Andres, M.I., Sánchez-Fortun, S., del Castillo, M.D. (2019). Validation of coffee by-products as novel food ingredients. *Innovative Food Science & Emerging Technologies, 51*, 194-204. https://doi.org/10.1016/j.ifset.2018.06.010.
- **Iriondo DeHond, A**., Abdolmaleky, H.M., Zhou, J.R., del Castillo, M.D. (2018). Assessment of antioxidant and anti-inflammatory effects of coffee by-product extracts on a cellular model of diabetic nephropathy, *Nutrición Clínica y Dietética Hospitalaria, 38*(supl. 1), 117.
- **Iriondo-DeHond, A**., Haza, A.I., Avalos, A., del Castillo, M.D., Morales, P. (2017). Validation of coffee silverskin extract as a food ingredient by the analysis of cytotoxicity and genotoxicity. *Food Research International, 100* (1), 791-797. https://doi.org/10.1016/j.foodres.2017.08.012.

Book chapters:

- **A. Iriondo-DeHond**, T. Herrera, M.D. del Castillo. (2020) Health benefits of silverskin. In *Food wastes and by-products: Nutraceutical & health potential.* (Ed.) R. Campos-Vega, Wiley-Blackwell. ISBN: 978-1-119-53410-5.

- M.D. del Castillo, **A. Iriondo-DeHond**, B. Fernandez-Gomez, N. Martinez-Saez, M. Rebollo-Hernanz, M.A. Martín-Cabrejas, A. Farah. (2019) Coffee antioxidants in chronic diseases. In *Coffee: Consumption and health implications.* (Ed.) A. Farah, Royal Society of Chemistry. ISBN: 978-1-78801-497-7.

- M.D. del Castillo, B. Fernandez-Gomez, N. Martinez-Saez, **A. Iriondo-DeHond,** M.D Mesa. (2019) Coffee by-products. In *Coffee: Production, quality and chemistry*. (Ed.) A. Farah, Royal Society of Chemistry. ISBN: 978-1-78801-497-7.

- **A. Iriondo-DeHond**, J. Uribarri, M.D. del Castillo. (2019) Usefulness of dietary components as sustainable nutraceuticals for chronic kidney disease. In *Encyclopedia of food security and sustainability.* (Ed.) P. Ferranti, E. Berry and A. Jock, Elsevier. ISBN: 9780128126875.

- M.D. del Castillo, **A. Iriondo-DeHond**, N. Martinez-Saez, B. Fernandez-Gomez, M. Iriondo-DeHond, J.R. Zhou. (2017) Applications of recovered compounds in food products. In *Handbook of Coffee Processing By-Products.* (Ed.) C. Galanakis, American Press. ISBN: 978-0-12-811290-8.

- M.D. del Castillo, B. Fernandez-Gomez, N. Martinez-Saez, **A. Iriondo-DeHond**, D.M. Martirosyan, M.D. Mesa. (2016) Coffee silverskin extract for aging and chronic diseases. In *Functional Foods for Chronic Diseases.* (Ed.) D.M. Martirosyan, CreateSpace Independent Publishing Platform. ISBN: 978-1536919431.

Other publications related to the PhD Thesis include:

— T. Herrera, **A. Iriondo-DeHond,** J. Uribarri, M.D. del Castillo. (2020) Beneficial herbs and spices. In *Nutrition, Fitness, and Mindfulness: An Evidence-Based Guide for Clinicians*. (Eds.) J. Uribarri and J.A. Vassalotti, Springer International Publishing AG. ISBN 978-3-030-30892-6.

— Fernández Fernández, A.M., **Iriondo DeHond, A**., Dellacassa, E., Medrano Fernandez, A., del Castillo, M.D. (2019) Assessment of antioxidant,

antidiabetic, antiobesity, and anti-inflammatory properties of a Tannat winemaking by-product. *European Food Research and Technology*, *245*(8), 1539-1551. https://doi.org/10.1007/s00217-019-03252-w

— del Castillo, M.D., **Iriondo-DeHond, A**., Martirosyan, D.M. (2018) Are Functional Foods Essential for Sustainable Health? *Annals of Nutrition and Food Science, 2* (1), 1-4.

— Dueñas Martín, M., **Iriondo-DeHond, A**., del Castillo, M.D. (2018) Efecto de los compuestos fenólicos en el metabolismo de los carbohidratos. *Revista Española de Nutrición Comunitaria; 24*(1).

— **Iriondo-DeHond, A**., Fernandez-Gomez, B., Martinez-Saez, N., Martirosyan, D.M., Mesa, M.D., del Castillo, M.D. (2017). Coffee silverskin: A low-cost substrate for bioproduction of high value health promoting products. *Annals of Nutrition and Food Science, 1* (1), 1-6.

— **Iriondo-DeHond, A**., Martorell, P., Genovés, S., Ramón, D., Stamatakis, K., Fresno, M., Molina, A., Del Castillo, M.D. (2016). Coffee silverskin extract protects against accelerated aging caused by oxidative agents. *Molecules, 1*;21(6). https://doi.org/10.3390/molecules21060721.

INDEX

ABBREVIATIONS

5-CQA	5-caffeoylquinic acid
AAA	Aromatic amino acids
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADA	American Dietetic Association
ALT	Alanine Aminotransferase
ANOVA	Analysis of variance
APCI	Atmospheric-pressure chemical ionization
ARE	Antioxidant response element
ATR	Attenuated total reflection
B(a)P	Benzo(a)pyrene
BAT	Bioanalytical Techniques Unit
BCAA	Branched-chain amino acids
BDNF	Brain derived neurotrophic factor
BHI	Brain heart infusion
BHT	Butylated hydroxytoluene
BPW	Buffered peptone water
BSA	Bovine serum albumin
BW	Body weight
CAF	Caffeine
CAT	Catalase
CEEA	Ethics Committee on Animal Use
CEL	N^{ε}-(carboxyethyl)lysine
CFU	Colony-forming unit
CGA	Chlorogenic acid
CIAL	Instituto de Investigación en Ciencias de la Alimentación
CIB	Biology Investigation Center
CML	N^{ε}-(carboxymethyl)lysine
CoC	Code of Conduct
COX-2	Cyclooxygenase-2
CRP	C - reactive protein
CS	Coffee silverskin
CSE	Coffee silverskin extract
CZE	Capillary zone electrophoresis
DAD	Diode array detector
DCF	Dichlorofluorescein
DCFH-DA	2',7'-dichlorodihydro-fluorescein diacetate
DDD	Dichlorodiphenyldichloroethane

DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAA	Essential amino acids
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
eLENA	e-Library of Evidence for Nutrition Actions
ELISA	Enzyme-linked immunosorbent assay
ENDO III	Endonuclease III
EOF	Electroosmotic flow
EU	European Union
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FDA	Food and Drug Administration from the United States of America
FL	N^{ε}-(fructosyl)lysine
FOS	Fructooligosaccharides
FPG	Formamidopyrimidine-DNA glycosylase
FT	Fourier-Transform
GGT	γ-glutamyl-transferase
GLP-1	Glucagon-like peptide type 1
GPx	Glutathione peroxidase
GR	Glutathione reductase
GRAS	Generally recognized as safe
HCH	Hexachlorocyclohexane
HCT	Hematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMW	High molecular weight
HPLC	High-performance liquid chromatography
IC50	Half maximal inhibitory concentration
IARC	International Agency for Research on Cancer
IDF	Insoluble dietary fiber
iNOS	Inducible nitric oxide synthase
IR	Infrared
LC-IC	Ion exchange liquid chromatography
LMP	Low melting point
LPS	Lipopolysaccharide
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration

MCV	Mean corpuscular volume
MEL	Melanoidins
MPV	Mean platelet volume
MS/MS	Tandem mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MUFA	Monounsaturated fatty acids
N.D.	Non determined
NMP	N-methylpyridinium
NO	Nitric oxide
NTU	Nephelometric turbidity units
OECD	Organization for Economic Co-operation and Development
ORAC	Oxygen radical absorbance capacity
OTA	Ochratoxin A
PAD	Pulsed Amperometric Detector
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PCA	Plate count agar
PPM	Parts per million
PUFA	Polyunsaturated fatty acids
QToF	Quadrupole-time of flight
RBCs	Red blood cells
RDAs	Recommended daily allowances
RDW	Red cell distribution width
ROS	Reactive oxygen species
RPM	Revolutions per minute
RUT	Rutin
SCFAs	Short-chain fatty acids
SCGs	Spent coffee grounds
SD	Standard deviation
SDA	Sabouraud dextrose agar
SDF	Soluble dietary fiber
SDH	Succinate dehydrogenase
SE	Standard error
SEM	Standard error of the mean
SFA	Saturated fatty acids
SIM	Selected ion monitoring
SOD	Superoxide dismutase
SPE	Solid phase extraction
STP	Society of Toxicologic Pathology
STZ	Streptozotocin
tBOOH	Tert-butyl hydroperoxide

TAE	Tris acetate EDTA
TE	Trolox equivalents
TDF	Total dietary fiber
TSS	Total soluble substances
TAC	Total antioxidant capacity
TDS	Total dissolved substances
TFC	Total flavonoid content
TNF	Tumor necrosis factor
TPC	Total phenolic content
UK	United Kingdom
UPLC	Ultra-performance liquid chromatography
URJC	Universidad Rey Juan Carlos
US	Unites States
USDA	US Department of Agriculture
UV	Ultraviolet
WBCs	White blood cells
WHO	World Health Organization

SUMMARY/RESUMEN

SUMMARY

The term "sustainable health" means "a healthy and active aging avoiding the risk of diseases". The fast-growing population, the increasing need for nutritionally correct diets and the depletion of food sources suppose an immense challenge for the global food system. In order to increase the eco-sustainability of the food processing industry, it is necessary to exploit co-products before they become wastes. The coffee industry is responsible for the generation of large amounts of residues that represent a pollution hazard if discharged into the environment. Dealing with these coffee waste recovery is of great importance to combat hunger, raise income and improve food security in the world's poorest countries.

The aim of the present PhD thesis was to validate the feasibility of coffee by-products (husk, parchment, silverskin and spent coffee grounds) as novel food ingredients for a sustainable nutrition and health following the Regulation (EU) 2015/2283. The nutritional composition and safety of coffee cherry by-products generated in producing countries (husk and parchment) were addressed. Further characterization, safety, nutritional value and health implications of ingredients from coffee by-products obtained worldwide (coffee silverskin and spent coffee grounds) were studied. The health promoting properties of isolated biomolecules from the latter were also evaluated.

By-products from coffee cherry processing were submitted to aqueous extraction, which is sustainable and easy to scale-up, for generating ingredients avoiding new wastes. Husk and silverskin were proposed as a source of two food ingredients: an aqueous extract enriched in phytochemicals and antioxidant dietary fiber. In contrast, parchment was proposed as a single source of antioxidant dietary fiber. Husk, parchment and silverskin resulted safe since the absence of pesticides, mycotoxins and lethal effects was observed in rats treated with by-products at 2000 mg/kg b.w.

Coffee silverskin extract (CSE) did not show cytotoxicity or genotoxicity. It also showed preventive effects against induced oxidative DNA damage. Results from the nutritional characterization of CSE showed that it might be considered a source of proteins (16 %), low in fat (0.44 %) and high in fiber (22 %). It can also be considered source of potassium, magnesium, calcium and vitamin C. Oral administration of CSE at a dose of 1 g/kg for a period of 28 days was not toxic to rats. Diet supplementation with CSE had no negative effects in hormone secretion, antioxidant or anti-inflammatory biomarkers. Total short chain fatty acids (SCFAs) derived from CSE fiber fermentation were significantly higher ($p < 0.05$) in male rats compared to male control rats.

The absence of mycotoxins and toxicity was also reported in spent coffee grounds (SCGs). A total of 77 % of unsaturated fatty acids and low amounts of kahweol (7.09 μg/g) and cafestol (414.39 μg/g) were bioaccessible after *in vitro* digestion. A significantly lower ($p < 0.1$) accumulation of lipids in the liver and a higher excretion of them in feces was found in rats treated with SCGs for 28 days. No lipid droplets or liver damage were observed by histology. SCGs acutely accelerated intestinal motility in rats. SCGs might be considered a sustainable, safe, and healthy food ingredient with potential for preventing hepatic steatosis due to their effect as dietary fiber with a high fat-holding capacity.

Multifunctional biomolecules for food industry, a fraction enriched in melanoidins, were isolated from CSE and SCGs and they showed antioxidant properties against oxidative stress induced in human colon cell lines. In addition, the fiber effect of melanoidins isolated from CSE was studied *in vivo* in healthy male Wistar rats. The isolation process was effective in obtaining a high molecular weight fraction, composed mainly of dietary fiber, including melanoidins. This fraction was administered to animals in the drinking water at a dose of 1 g/kg and after 28 days, no signs of toxicity were observed and gastrointestinal motility was significantly accelerated ($p < 0.05$) compared to the control group.

In conclusion, findings derived from the present PhD thesis confirmed the feasibility of using coffee by-products as novel food ingredients. The most preferred application for coffee husk and silverskin is as two food ingredients for human consumption. However, parchment is proposed to be used directly for plastic replacement in intelligent food packaging. The high molecular weight fraction enriched in melanoidins isolated from coffee silverskin can be used for technological (natural colorant) or for nutritional and health (dietary fiber effect) purposes. The most efficient use of instant SCGs seems to be as a food ingredient high in dietary fiber. Crucial data for the application of the Novel Food Authorization for husk, CSE and SCGs to the European Commission have been obtained in this investigation. The present investigation represents a significant contribution to the sustainability of the coffee industry and to achieve a sustainable nutrition and health.

RESUMEN

El término "salud sostenible" significa "un envejecimiento saludable y activo que previene el riesgo de enfermedades". El rápido crecimiento de la población, la creciente necesidad de dietas nutricionalmente correctas y el agotamiento de las fuentes de alimentos suponen un desafío inmenso para el sistema alimentario mundial. Para aumentar la sostenibilidad de la industria alimentaria, es necesario explotar los co-productos antes de que se conviertan en desechos. La industria del café es responsable de la generación de grandes cantidades de residuos que representan un peligro de contaminación si se desechan al medio ambiente. Buscar nuevas aplicaciones para estos desechos es de gran importancia para combatir el hambre, aumentar los ingresos y mejorar la seguridad alimentaria en los países más pobres del mundo.

El objetivo de la presente tesis doctoral fue validar la viabilidad de los subproductos del café (cáscara, pergamino, cascarilla y posos) como nuevos ingredientes alimentarios para una nutrición y salud sostenible siguiendo el Reglamento (UE) 2015/2283. Por un lado, se analizó la composición nutricional y la seguridad alimentaria de los subproductos de la cereza del café generados en los países productores (cáscara y pergamino). Por otro lado, se estudió la composición nutricional y seguridad alimentaria de los subproductos del café obtenidos en los países consumidores (cascarilla y posos). Finalmente, se realizó una evaluación de las propiedades beneficiosas para la salud de las biomoléculas aisladas de los estos últimos.

Los subproductos del procesamiento de la cereza de café se sometieron a extracción acuosa, que es sostenible y fácilmente escalable, para generar ingredientes evitando la generación de nuevos desechos. La cáscara y cascarilla se propusieron como fuente de dos ingredientes alimentarios: un extracto acuoso enriquecido en fitoquímicos y fibra dietética antioxidante. Por el contrario, se propone el uso del pergamino como una única fuente de fibra dietética antioxidante. La cáscara, el pergamino y la cascarilla resultaron seguros ya que se observó la ausencia de pesticidas, micotoxinas y efectos letales en ratas tratadas con los subproductos a 2000 mg/kg de peso corporal.

El extracto de cascarilla de café (CSE) no mostró citotoxicidad ni genotoxicidad. Además, mostró efectos preventivos contra el daño oxidativo inducido en el ADN. Los resultados de la caracterización nutricional de CSE mostraron que podría considerarse una fuente de proteínas (16 %), baja en grasas (0,44 %) y alta en fibra (22 %). También puede considerarse fuente de potasio, magnesio, calcio y vitamina C. La administración oral de CSE a una dosis de 1 g/kg durante un período de 28 días no fue tóxica para las ratas. La suplementación dietética con CSE no tuvo efectos negativos en la secreción

hormonal, biomarcadores antioxidantes o antiinflamatorios. Los ácidos grasos de cadena corta (SCFAs) totales derivados de la fermentación de la fibra de CSE fueron significativamente mayores ($p < 0,05$) en ratas macho en comparación con las ratas macho control.

La ausencia de micotoxinas y de toxicidad también se confirmó en los posos de café (SCGs). El 77 % de ácidos grasos insaturados y bajas cantidades de kahweol (7.09 μg/g) y cafestol (414.39 μg/g) fueron bioaccesibles después de la digestión *in vitro*. Se encontró una acumulación significativamente menor ($p < 0,1$) de lípidos en el hígado y una mayor excreción de estos en las heces de ratas tratadas con SCGs durante 28 días. No se observaron gotas lipídicas ni daño hepático. Los SCGs aceleraron de forma aguda la motilidad intestinal en ratas. Los SCGs pueden considerarse un ingrediente alimentario sostenible, seguro y saludable con potencial para prevenir la esteatosis hepática debido a su efecto como fibra dietética con una alta capacidad de retención de grasa.

Biomoléculas multifuncionales para la industria alimentaria, una fracción enriquecida en melanoidinas, se aislaron de CSE y SCGs y mostraron propiedades antioxidantes contra el estrés oxidativo inducido en líneas celulares de colon humano. Además, el efecto fibra de melanoidinas aisladas de CSE se estudió *in vivo* en ratas Wistar macho sanas. El proceso de aislamiento fue efectivo para obtener una fracción de alto peso molecular, compuesta principalmente por fibra dietética, que incluye melanoidinas. Esta fracción se administró a animales en el agua de bebida a una dosis de 1 g/kg y después de 28 días, no se observaron signos de toxicidad y la motilidad gastrointestinal se aceleró significativamente ($p < 0,05$) en comparación con el grupo de control.

En conclusión, los resultados derivados de la presente tesis doctoral confirmaron la viabilidad del uso de los subproductos del café como nuevos ingredientes alimentarios. La mejor aplicación para la cáscara y cascarilla es como dos ingredientes alimentarios para el consumo humano. Sin embargo, el pergamino podría ser utilizado directamente para el reemplazo de plástico en el envasado inteligente de alimentos. La fracción de alto peso molecular enriquecida en melanoidinas aisladas de la cascarilla de café se puede usar con fines tecnológicos (colorantes naturales) o con fines nutricionales y de salud (efecto de fibra dietética). El uso más eficiente de los SCGs instantáneos es como ingrediente alimentario rico en fibra dietética. En esta investigación se obtuvieron datos cruciales para la autorización de Nuevos Alimentos para cáscara, CSE y SCGs a la Comisión Europea. La presente investigación representa una contribución significativa a la sostenibilidad de la industria del café y una nutrición y salud sostenibles.

INTRODUCTION

1. Sustainable nutrition and health framework

"A healthy and active ageing avoiding the risk of diseases" is the definition for "sustainable health", which can be achieved through a healthy nutrition (María Dolores del Castillo, Iriondo-DeHond, & Martirosyan, 2018). A sustainable nutrition and health is based on "sustainable diets", defined by the Food and Agriculture Organization (FAO) as "those diets with low environmental impacts which contribute to food and nutrition security and to healthy life for present and future generations. Sustainable diets are protective and respectful of biodiversity and ecosystems, culturally acceptable, accessible, economically fair and affordable; nutritionally adequate, safe and healthy; while optimizing natural and human resources" (FAO & Food Climate Research Network, 2016).

A sustainable and healthy nutrition is a research priority in the FOOD 2030 team of the European Commission, which is a European Union (EU) Research & Innovation policy framework that attempts to future-proof our nutrition and food systems for a sustainable food production and consumption. In this context, its aim is to ensure that nutritious food and water is available, accessible and affordable for all. This involves reducing hunger and malnutrition, ensuring high levels of food safety and traceability, reducing the incidence of non-communicable diet-related diseases and helping all citizens and consumers adopt sustainable and healthy diets for good health and wellbeing (European Commission, 2018).

The fast-growing population, the increasing need for nutritionally correct diets and the depletion of food sources suppose an immense challenge for the global food system. A potential strategy to achieve a sustainable nutrition and health is Bioeconomy, or circular economy, that means "the production and utilization of biological resources, biological processes and principles to sustainably provide goods and services across all economic sectors" (Food and Agriculture Organization of the United Nations (FAO), 2018).

In order to increase the eco-sustainability of the food processing industry, it is necessary to exploit co-products before they become wastes. Food losses and wastes have an impact on food security, on food quality and safety, on economic development and on the environment. Dealing with this food waste is of great importance to combat hunger, raise income and improve food security in the world's poorest countries (Food and Agriculture Organization of the United Nations (FAO), 2011). Until a few decades ago, food wastes were considered neither a cost nor a benefit, they were used as animal feed or brought to landfills or sent for composting (Kumar, Yadav, Kumar, Vyas, & Dhaliwal, 2017). However, to create the most benefits for the environment, society and economy,

FAO is currently developing a Code of Conduct (CoC) for the reduction of food loss and food waste and has proposed the guidance illustrated in Figure 1 (Food and Agriculture Organization of the United Nations (FAO), 2019).

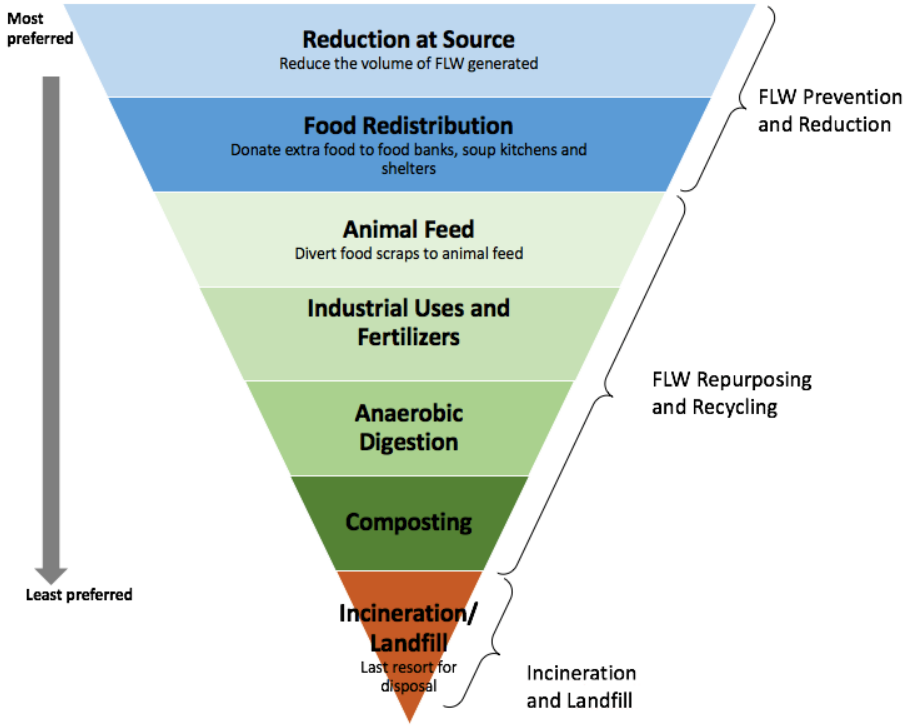


Figure 1. The food hierarchy proposed by the Food and Agriculture Organization of the United Nations (FAO, 2019). FLW, Food losses and waste.

This inverted pyramid sets priorities on how best reduce food waste and save natural resources. The first recommendation is to avoid generating waste in the first place. Then, when prevention of waste cannot be done, excess of food should be donated to people in need. The next option is to use food waste in animal feeding, fuel conversion, composting, and finally, incineration is the least preferred option. In this context, the present PhD thesis proposes to use food by-products as novel ingredients within the food sector with the final aim to contribute to a sustainable nutrition and health.

2. Novel ingredients for a sustainable nutrition and health

In order to ensure the protection of human health, specific legislation (Regulation (EU) 2015/2283) must be applied to food waste or by-products before their placement as novel food ingredients on the European Union market. According to this regulation, 'Novel Food' is defined as "food that had not been consumed to a significant degree by humans in the EU before 15 May 1997, when the first Regulation on novel food came into force" (EFSA Panel on Dietetic Products, 2016).

In this context, an application processed by the European Commission is necessary for its authorization. First, administrative data regarding a table of contents, company or organization of the applicant and a contact person must be included in 'Part 1' of the application (EFSA Panel on Dietetic Products, 2016). Then, requirements related to the characterization of the novel food ('Part 2') that should be included in all applications are summarized in Figure 2.

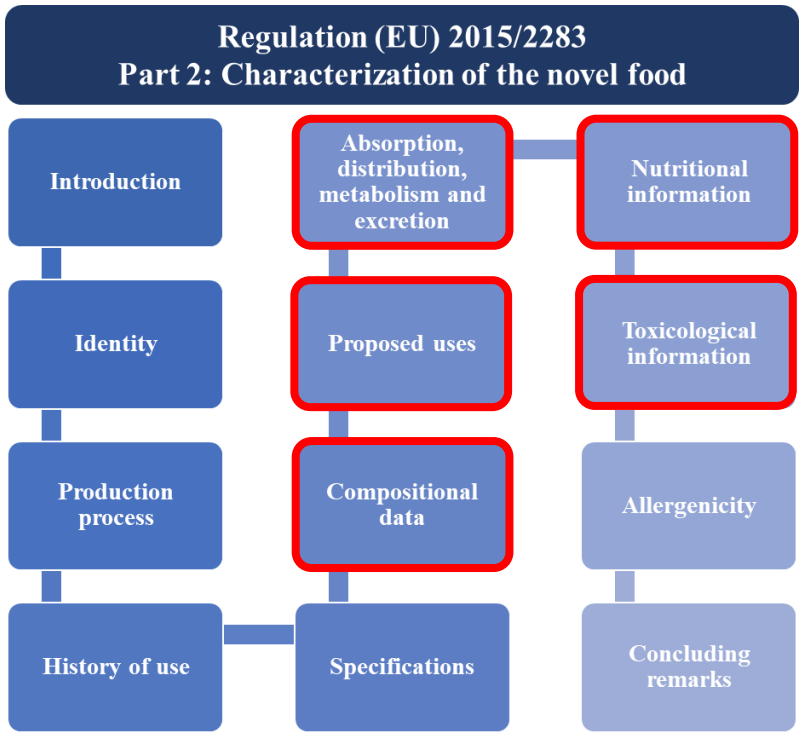


Figure 2. Summary of the requirements needed to be included in the applications for the authorization of a novel food under the Regulation (EU) 2015/2283. Highlighted sections will be addressed in the present PhD thesis.

The valorization of food wastes can be carried out through an integrated bio-refinery approach, in order to produce bioactive molecules for pharmaceutical, cosmetic, food, and non-food applications. Food waste reduction and valorization can be achieved through the extraction of high-value components such as proteins, polysaccharides, fibers, flavor compounds and phytochemicals, which can be re-used as novel food ingredients (Baiano, 2014). This potentially marketable components present in foods wastes and by-products need to be separated from the matrix through different extraction processes for selective extraction of the targeted components. In the end, the whole by-product should be re-used in order not to generate any more wastes. The extraction must be economically feasible and must avoid microbiological hazards (Baiano, 2014). In this investigation, aqueous extraction processes are proposed since they are green processes that meet the challenges to protect both the environment and consumers.

There is growing interest of consumers towards bioactive compounds present in foods that provide beneficial effects to humans in terms of health promotion and disease risk reduction (Kumar et al., 2017). Therefore, the incorporation of novel ingredients in foods could make them reach a nutrition or health claim. According to the European Commission, a "Nutrition claim" means "any claim which states, suggests or implies that a food has particular beneficial nutritional properties due to the energy (calorific value) it provides or does not provide; or to the nutrients or other substances it contains or does not contain" (The European Parliament and The Council of the European Union, 2006). Nutrition claims are only permitted if they are listed in the Annex of Regulation (EC) No 1924/2006.

Further beneficial health promoting properties of novel ingredients obtained from food wastes can be indicated by "Health claims", which are any statement about a relationship between food and health (The European Commission, 2012). There are three types of health claims, function health claims, risk reduction claims and those referring to children's development. The Commission authorizes submitted health claims that are based on scientific evidence, evaluated by the European Food Safety Authority (EFSA), and that can be easily understood by consumers. The list of permitted health claims is in the Commission Regulation (EU) 432/2012 and it is constantly updated with newly authorized health claims.

The formulation of foods with nutrition and health claims containing novel ingredients from by-products generated from food processing is an interesting strategy to contribute to a sustainable nutrition and health of the world's population.

3. Coffee by-products as novel ingredients

Coffee is one of the most popular beverages consumed all over the world. The most important plant species of the international coffee trade are *Coffea arabica* L. and *Coffea canephora*, which belong to the genus *Coffea* of the Rubiaceae family (Alves, Rodrigues, Nunes, Vinha, & Oliveira, 2017). To obtain the coffee beverage, approximately 90 % of the edible parts of the coffee cherry are discarded as an agricultural waste or by-product (Esquivel & Jiménez, 2012). Coffee by-products have attracted great attention because of their abundance and interesting chemical composition.

3.1. Definition and composition

The coffee cherry consists of an outer skin or pericarp, usually green in unripe and red in ripe fruits, that covers a soft and sweet pulp or outer mesocarp (Figure 3). This is followed by a viscous and highly hydrated layer of mucilage (pectin layer), a thin yellowish endocarp, the parchment; and finally, the silverskin that covers each hemisphere of the green coffee bean (endosperm) (Esquivel & Jiménez, 2012).

Figure 3. Anatomic parts of a ripe coffee cherry.

3.1.1. Husks

The processing of coffee initiates with the conversion of coffee cherries into green coffee beans, which consists of the removal of the skin and pulp using either a wet or dry method (Figure 4) (Mussatto, Machado, Martins, & Teixeira, 2011).

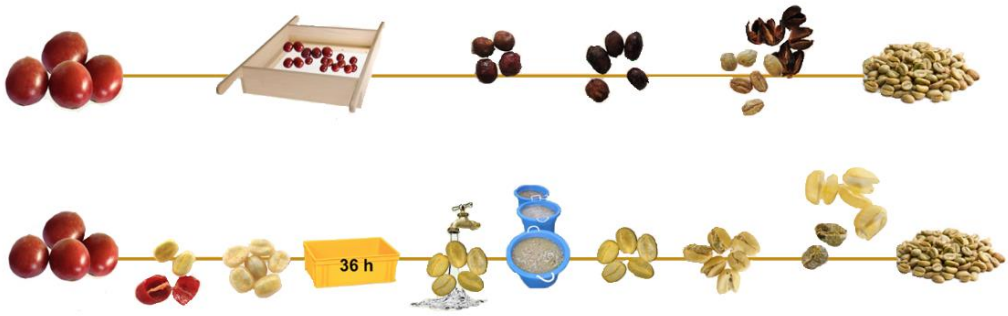


Figure 4. Dry (top) and wet (bottom) processing of coffee cherries. Figure adapted from (De Bruyn et al., 2017).

The dry method, commonly used in Robusta producing countries (Figure 5), is technologically simpler. The freshly harvested cherries are spread evenly and are dried for 2 – 4 weeks under the sun in yards (natural drying) or in mechanical dryers until moisture is below 12 %. Then, the cherries are mechanically dehusked and the skin, pulp and parchment are removed from the beans (Figure 4) (Alves et al., 2017; Murthy & Madhava Naidu, 2012). Coffee husks obtained from the dry method are composed by the outer skin, pulp and parchment and comprise nearly 45 % of the coffee cherry (M. Dolores del Castillo, Fernandez-Gomez, Martinez-Saez, Iriondo-DeHond, & Mesa, 2019).

In the wet process, commonly used for Arabica that is commercially more appreciated, the skin and pulp covering the beans are removed by a depulper (Murthy & Madhava Naidu, 2012). After the depulping process, the bean is still covered by the mucilage which is eliminated by fermentation for 24 to 72 h (Alves et al., 2017). Then, the bean covered by the parchment is washed, drained and dried until the moisture reaches around 10 % (Murthy & Madhava Naidu, 2012). Finally, the parchment is removed using hulling machinery (Figure 4) (Esquivel & Jiménez, 2012).

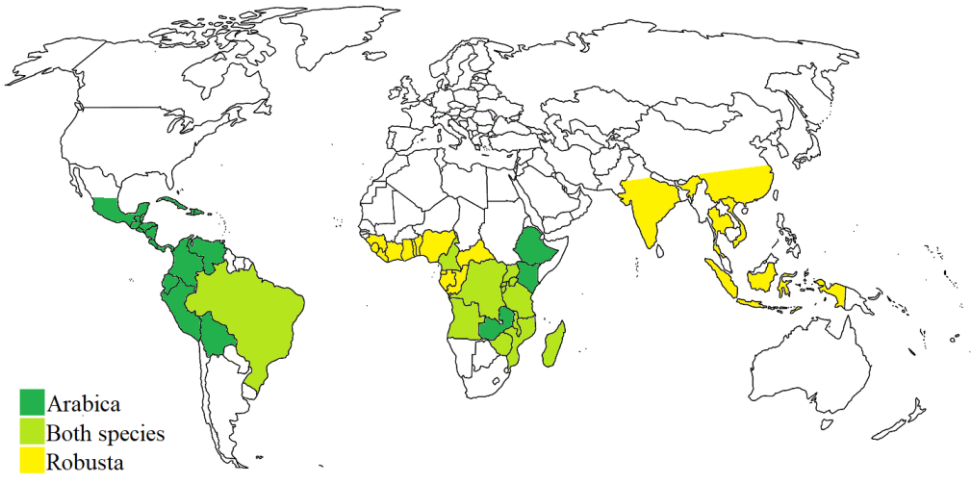


Figure 5. Map of production of Arabica and Robusta coffee. In countries were Arabica is produced, the wet processing method is employed. In Robusta producing countries, the dry method is carried out.

Coffee husks generated from the wet coffee processing (Figure 6) consist of the skin and pulp and represent 29 % dry weight of the whole bean (Murthy & Madhava Naidu, 2012). These coffee by-products are generated in coffee producing countries and constitute a source of severe contamination and a serious environmental problem. For instance, in Colombia, one of the first producers of Arabica coffee (International Coffee Organization, 2019), from 100 kg of mature coffee cherries, 39 kg correspond to skin and pulp (Ferrão, 2009).

Figure 6. Coffee husks obtained by the wet processing method.

Coffee husks from the dry and wet processing have a similar content of carbohydrates, fiber, minerals and proteins (Table 1) (Brand et al., 2001; Gouvea, Torres, Franca, Oliveira, & Oliveira, 2009). Since the chemical composition of husks obtained by the two different methods of processing (dry and wet) is somehow similar, in the following sections, the husks obtained by one method or the other will not be distinguished.

Table 1. Chemical composition of husks from the dry and wet processing. Data from [1](Oliveira & Franca, 2014) and [2](Janissen & Huynh, 2018).

	Husks from the dry processing	**Husks from the wet processing**
Carbohydrates (%)[1]	58 – 85	45 – 89
Total fiber (%)[2]	32	18 – 21
Lipids (%)[1]	0.5 – 3	1 – 2
Protein (%)[1]	8 – 11	4 – 12
Minerals (%)[1]	3 – 7	6 – 10
Caffeine (%)[1]	1	1

Coffee husks from the dry processing (skin, pulp and parchment), are also rich in insoluble dietary fiber, containing 24.5 % cellulose, 29.7 % hemicelluloses and 23.7 % lignin (Bekalo & Reinhardt, 2010). They are also a source of phytochemicals such as tannins (5 – 9 %) and cyanidins (20 %) (Brand et al., 2001; Gouvea et al., 2009; Prata & Oliveira, 2007). The amount of total polyphenols in coffee husks is 1.22 % (Murthy & Madhava Naidu, 2012). Similarly, husks obtained by the wet method (skin and pulp) also contain appreciable amounts of polyphenols (1.48 %) and caffeine (1.3 %) (Brand et al., 2001; Pandey et al., 2000; Porres, Alvarez, & Calzada, 1993).

Four major classes of polyphenols have been described in the husks obtained from the wet processing of *C. arabica* L. beans: flavan-3-ols, hydroxycinnamic acids, flavonols and anthocyanidins (Ramirez-Coronel et al., 2004). Chlorogenic acid and protocatechuic acid are the main polyphenols in coffee husks and represent more than 80 % of the polyphenols analyzed by Heeger *et al*. (2017) (Heeger, Kosińska-Cagnazzo, Cantergiani, & Andlauer, 2017). The composition of phenolic compounds in fresh husks has been analyzed by HPLC, and the obtained profile was chlorogenic acid (5-caffeoylquinic acid) (42.2 % of total identified phenolic compounds), epicatechin (21.6%), 3,4-dicaffeoylquinic acid (5.7 %), 3,5-dicaffeoylquinic acid (19.3 %), 4,5-dicaffeoylquinic acid (4.4 %), catechin (2.2 %), rutin (2.1 %), protocatechuic acid (1.6 %) and ferulic acid (1.0 %) (Ramirez-Martinez, 1988). The major anthocyanins present

in the husks derived from wet-processed fruits are cyanidin-3-rutinoside, cyanidin-3-glucoside and aglycone (Esquivel & Jiménez, 2012).

3.1.2. Mucilage

The coffee mucilage fraction (Figure 7) is located between the pulp and the parchment and represents 5 % dry weight of the cherry (Figure 3). It remains adhered to the coffee bean after depulping in the wet processing and since it is highly hydrated, it is an obstacle to further drying the beans. Thus, mucilage must be degraded by fermentation and washing, before the beans are dried and stored (Figure 4) (Avallone, Guiraud, Guyot, Olguin, & Brillouet, 2000). Wet processing allows the separation and concentration of this fraction, and therefore, this by-product is obtained in coffee producing countries that grow Arabica coffee (Esquivel & Jiménez, 2012). In Colombia, from 100 kg of fresh coffee cherries, 22 kg of mucilage are generated (Ferrão, 2009).

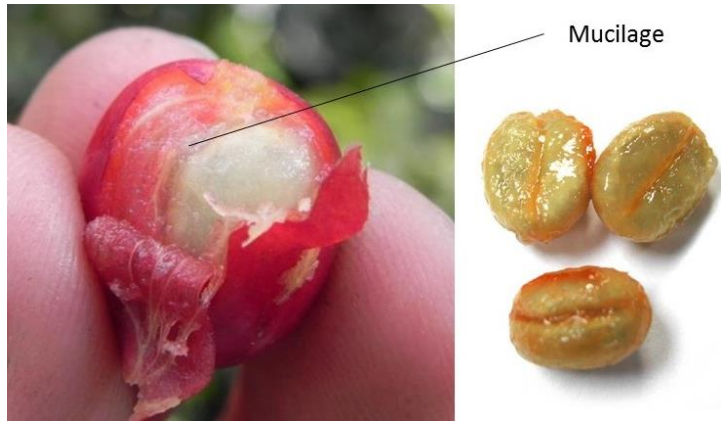


Figure 7. Ripped open coffee cherry, showing coffee pulp and mucilage. Figure from del Castillo *et al.* (2019).

Mucilage is composed of water (84.2 %), protein (8.9 %), sugar (4.1 %), pectic substances (0.91 %) and ash (0.7 %) (Esquivel & Jiménez, 2012). Recent studies have reported in coffee mucilage values of 3.71 %, 1.47 % and 0.08 % of glucose, galactose and lactose, respectively (Orrego, Zapata-Zapata, & Kim, 2018). Micronutrient composition of coffee mucilage was calcium (337 ppm), iron (73 ppm), magnesium (81 ppm), potassium (0.016 %), phosphorous (115 ppm) and sodium (29 ppm) (Orrego et al., 2018).

3.1.3. Parchment

This yellowish by-product is a strong fibrous endocarp that covers both hemispheres of the coffee seed and separates them from each other (Figures 3 and 8). It represents 5.8 % dry weight of the cherries. In the dry process, coffee parchment is separated from the green bean together with the skin and the pulp. This by-product containing all the different parts of the coffee cherry is generated in countries were mainly Robusta coffee is cultivated (Figure 5). In contrast, in the wet processing, the parchment is removed after drying and hulling in separate steps (Figure 4). The latter process allows the parchment to be collected in countries were Arabica coffee is produced (Figure 5) and used separately from other by-products (Belitz, Grosch, & Schieberle, 2009). In Colombia, the main Arabica coffee producer, from 100 kg of fresh coffee cherries, 39 kg of parchment are generated during processing (Ferrão, 2009).

Figure 8. Coffee parchment obtained by the wet method.

Coffee parchment is mainly constituted by dietary fiber, which ranged from 89 to 91 % (Benitez et al., 2019). The dietary fiber of coffee parchment is solely composed by insoluble fiber, corresponding to (α-) cellulose (40 – 49 %), hemicellulose (25 – 32 %), lignin (33 – 35 %) and ash (0.5 – 1 %) (Bekalo & Reinhardt, 2010).

3.1.4. Silverskin

Green beans are then exported to coffee consuming countries and their quality is evaluated based on odor and taste tests, as well as on the size, shape, color, hardness and presence of defects (Mussatto, Machado, et al., 2011). After reception and confirmation

of their quality, the beans are stored until roasting. The roasting process can be divided into three consecutive stages: drying, roasting and cooling. During the drying stage, water and volatile substances are slowly released and the coffee beans change their color from green to yellow. Roasting reactions produce changes in both physical and chemical properties of beans and results in the generation of a complex mixture of Maillard reaction products (Murthy & Madhava Naidu, 2012). At this stage, the silverskin (the thin tegument that covers the bean) is detached and represents the only by-product of coffee roasting industry (Figure 9) (Alves et al., 2017).

Coffee silverskin (CS) is a thin tegument of the outer layer of the two beans forming the green coffee seed (Figure 3) obtained as a by-product of the roasting process produced by large-scale coffee roasters in consuming countries (Mussatto, Machado, et al., 2011). It represents about 4.2 % (w/w) of coffee beans (Narita & Inouye, 2014).

Figure 9. Coffee silverskin produced during the roasting process of green coffee beans.

The roasting of 4 tons of coffee produces around 30 kg of silverskin (A. S. G. G. Costa et al., 2014). Considering that world coffee production for 2019/20 is forecast to 169.1 million 60 kg bags (USDA Foreign Agricultural Service, 2019), 76,095 tons of CS will be produced next year all over the world in coffee transforming countries; leading to the consequent environmental problems. In Spain, the roasting of 120 kg of coffee generates 2.5 kg of silverskin (data provided by the company Supracafé S.A.).

CS has high dietary fiber content (68 – 80%), which includes about 85 % insoluble dietary fiber and 15 % soluble dietary fiber (Borrelli, Esposito, Napolitano, Ritieni, & Fogliano, 2004; Toschi, Cardenia, Bonaga, Mandrioli, & Rodriguez-estrada, 2014). Insoluble dietary fiber is composed by glucose, xylose, galactose, mannose and arabinose that are polymerized into cellulose and hemicellulose structures (Borrelli et al., 2004; Pourfarzad, Mahdavian-Mehr, & Sedaghat, 2013). Lignin is also a fraction present in a significant amount in CS (30 %) (Ballesteros, Teixeira, & Mussatto, 2014). The composition of sugars depends on the process used to extract carbohydrates and varies greatly in this by-product (1.6 – 12 %). Some studies have found glucose to be the main monosaccharide in CS while others reported fructose to be the main monosaccharide (Ballesteros et al., 2014; Toschi et al., 2014). CS contains 16.2 – 19.0 %, 1.56 – 3.28 % and 5 – 7 % of protein, fat and ash, respectively (Borrelli et al., 2004; Jiménez-Zamora, Pastoriza, & Rufián-Henares, 2015; Napolitano, Fogliano, Tafuri, & Ritieni, 2007; Pourfarzad et al., 2013). The ash in CS contains a variety of mineral elements including potassium, calcium, magnesium, sulfur, phosphorus, iron, manganese, boron and copper among others. Potassium is the most abundant mineral element followed by calcium and magnesium (Ballesteros et al., 2014). Caffeine content in CS is 0.8 – 1.4 %, which is slightly lower than what is present in coffee beans (1.2 - 2.5 %) (Farah, 2012; Napolitano et al., 2007; Toschi et al., 2014). Both in the coffee beans and in CS, the higher levels of caffeine are found in Robusta compared to Arabica.

CS is considered to be a good source of non-nutrients bioactive compounds, particularly chlorogenic acids. The most relevant are 5-caffeoylquinic acids and 3-caffeoylquinic acids with amounts of 1.99 mg/g and 1.48 mg/g, respectively (Bresciani, Calani, Bruni, Brighenti, & Del Rio, 2014; Murthy & Naidu, 2012). As expected from being a by-product generated during the roasting process, the presence of melanoidins has also been reported in CS (Borrelli et al., 2004). Melanoidins are the final product of the Maillard reaction, which occurs during the roasting process of the coffee bean. Mainly polysaccharides, proteins and chlorogenic acid form coffee melanoidins. Different conditions of roasting give rise to the formation of different types and amounts of melanoidins (Moreira, Nunes, Domingues, & Coimbra, 2012).

3.1.5. Spent coffee grounds

Finally, roasted beans are ground and the coffee beverage is prepared to get the most flavor in a cup of coffee. There are many different ways of preparing the coffee beverage: decoction (boiled coffee, Turkish coffee, percolator coffee), infusion (filtered and napoletana coffees) and pressure (press-pot, mocha, and espresso coffees) (Alves et al.,

2017). In addition, almost 50 % of the world coffee production is processed for soluble/instant coffee (Esquivel & Jiménez, 2012). The last coffee by-product, spent coffee grounds (SCGs), is generated during coffee brewing in coffee transforming countries. They obtained during the elaboration of the coffee beverage employing traditional methods and during instant coffee production (Figure 10). One ton of green coffee generates about 650 kg of SCGs, and about 2 kg of wet SCGs are obtained for each kilogram of soluble coffee produced, with an annual generation of around 6 million tons worldwide (Mussatto, Machado, et al., 2011).

Figure 10. Spent coffee grounds obtained by traditional or industrial brewing processes.

Polysaccharides are the main components of SCGs derived from instant coffee production (75 %). Hemicellulose (39 %) and cellulose (12 %) are the most abundant polysaccharides in SCGs. Sugars composing SCGs are 37 % mannose, 32 % galactose, 24 % glucose and 7 % arabinose (Ballesteros et al., 2014). The dietary fiber content in SCGs is 43 – 54 % total dietary fiber, 47 – 50% insoluble dietary fiber and 6 – 16 % soluble dietary fiber. SCGs also contain protein, fat and ash (13.6 – 17.44 %, 10 - 29 % and 1.3 –1 .6 %, respectively) (Ballesteros et al., 2014; Rocio Campos-Vega, Loarca-Piña, Vergara-Castañeda, & Oomah, 2015; Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011). With regard to minerals, potassium is the major component, followed by magnesium and phosphorus (Ballesteros et al., 2014). Various caffeine concentrations (0.007 – 0.5 %) have been reported depending on the caffeine extraction process and SCGs variety (Andrade et al., 2012; Ramalakshmi, Rao, Takano-Ishikawa, & Goto, 2009). SCGs from different coffeemakers (filtered, French press and espresso) contained between 0.2 and 0.8 % caffeine with the exception of SCGs from the regular coffeemaker, which did not present this compound (Bravo, Jua, Monente, Caemmerer,

& Kroh, 2012). Differences in chemical composition of SCGs probably reflect the variety of beans and processes used in roasting and extraction (Rocio Campos-Vega et al., 2015).

Chlorogenic acids, such as monocaffeoylquinic acids (3-CQA, 4-CQA, 5-CQA) and dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, 4,5-diCQA) have been identified and quantified in SCGs obtained from different brewing processes and coffee species (Bravo et al., 2012; Jiménez-Zamora et al., 2015). Levels of total caffeoylquinic acids (CQA) reported in SCGs were from 0.7 to 1.7 % and they were higher in Arabica SCGs than in Robusta SCGs (Bravo et al., 2012; Jiménez-Zamora et al., 2015). Coffee melanoidins remained in SCGs after coffee brewing and ranged from 15 to 35 % (Jiménez-Zamora et al., 2015). Coffee melanoidin fractions in SCGs are diverse and possess different physico-chemical properties (Moreira et al., 2012).

3.2. Nutritional value

Humans require a wide range of essential nutrients for a normal development and to support a healthy aging (US Department of Agriculture, 2015). Improving diets and increasing levels of physical activity in adults and older people will reduce the risk of chronic diseases (WHO, 2003). There is a consensus on the guidance of the meaning of a "balanced diet" described in terms of the proportions of the various energy sources, from diet in relation to a healthy lifestyle and the prevention of chronic diseases. Recommendations are as follows: 55 – 75 % of total energy should come from carbohydrates, 15 – 30 % from fat and 10 – 15 % from protein (WHO, 2003). In this context, coffee by-products have been proposed as a potential sustainable source of macro, micronutrients and non-nutrient bioactive compounds.

Table 2 summarizes the nutrition claims attributed to each coffee byproduct. Both coffee husks obtained from the dry and wet processing can be considered "high in fiber" since they contain more than 6 % of dietary fiber (Table 2). This by-product could contribute to the recommended intake of 25 g of dietary fiber per day made by the EFSA (European Food Safety Authority (EFSA), 2010). One of the most interesting characteristic of coffee husks is their content in soluble dietary fiber. This type of dietary fiber can be fermented leading to the production of short chain fatty acids (SCFAs) with health promoting properties, and also possesses large water retention, promotes the growth of bifidobacteria and decreases the absorption of fat and sugars (Ballesteros et al., 2014).

Table 2. Location of generation, step of processing, nutrition claims and health promoting properties of coffee by-products.

By-product	Location	Step of processing	Nutrition claims	Health promoting properties	References
Husks	Producing countries	Green bean obtainment	High in fiber	Anti-diabetic Antioxidant Anti-inflammatory	Murthy et al., 2012; Iriondo-DeHond, 2019 Secret Cherry Tea, 2019; Iriondo-DeHond, 2019 Magoni et al., 2018, Rebollo-Hernanz et al., 2019
Mucilage	Producing countries	Green bean obtainment	-	Antioxidant	Natucafé S.A.S., 2019
Parchment	Producing countries	Green bean obtainment	High in fiber	Hypoglycemic Hypolipidemic	Benitez et al., 2019
Silverskin	Transforming countries	Roasting	High in fiber Source of proteins Low in fat	Prebiotic Anti-obesity Anti-diabetic Antioxidant Anti-inflammatory Skin health	Borrelli et al., 2004 Martinez-Saez et al., 2014 WO/2016/097450; Fernandez-Gomez et al., 2016 Fernandez-Gomez et al., 2016 Rebollo-Hernanz et al., 2019 WO/2013/004873; Rodrigues et al., 2016
Spent coffee grounds	Transforming countries	Brewing	High in fiber Source of proteins	Prebiotic Antioxidant Anti-inflammatory	Jimenez-Zamora et al., 2015 Martinez-Saez et al., 2017 Lopez-Barrera et al., 2016

Different companies are already using coffee husks as a source of dietary fiber. Pectcof B.V. extracts soluble dietary fiber from coffee husks for both food and non-food applications. Due to its properties as an emulsifier and stabilizer, this extracted coffee dietary fiber is a promising new ingredient for the food and beverage industry ("Pectcof B.V.," 2019). On the other hand, Ramirez Velez and Jaramillo Lopez patented in 2015 the extraction of honey or flour from coffee husks for its use in products for human or animal consumption, drugs and cosmetics or as raw materials for the production of alcohol for fuel (ethanol) (Ramirez Velez & Jaramillo Lopez, 2015). In addition, The Coffee Cherry Co. also uses coffee husks flour as a healthy and sustainable ingredient in different food formulations such as breads, cookies, muffins, squares, brownies, pastas, sauces and beverages. This flour is gluten-free, it possesses five times more fiber than wholegrain wheat flour, contains antioxidants, iron, potassium and proteins and has low fat content ("The Coffee Cherry Co.," 2014).

According to the International Coffee Organization, coffee mucilage could be used in foods as an unrefined source of pectin, antioxidants and flavonoids. All these compounds have raised special interest in the food industry (Rathinavelu & Graziosi, 2005). Coffee mucilage has also been proposed for its use as a honey for human consumption (Ramirez Velez & Jaramillo Lopez, 2015). The chemical composition of this honey showed 30 – 40% moisture, 55 °Bx, 4 % proteins, 2 % fiber and a polyphenol content corresponding to 380 mg gallic acid equivalents/100 g. This coffee honey with high sugar content was achieved by means of a vacuum dehydration step at a temperature below 65 °C, obtaining a product with minimum nutritional damage by heat, and high digestibility and palatability (Ramirez Velez & Jaramillo Lopez, 2015).

With regard to coffee parchment, considering the chemical composition of this by-product, it may be used as a natural source of dietary fiber in the food industry since it can be considered "high in fiber". A very recent study has evaluated coffee parchment as a potential dietary fiber ingredient (Benitez et al., 2019). Results revealed that coffee parchment (flakes and flour) was a good source of insoluble dietary fiber (IDF), mainly composed by xylans (35%), lignin (32%), and cellulose (12%).

Considering the chemical composition of CS, the following nutrition claims could be applied to this by-product: "source of proteins" and "low in fat" since it contains over 12 % proteins and less than 3 % fat, respectively (Table 2) (European Commission, 2012). Another nutrition claim suitable for CS is "high in fiber". Fiber-enriched foods have been developed in the past few years to increase dietary fiber consumption to reduce the risk of chronic diseases (Ateş & Elmacı, 2018b). CS has been employed as

dietary fiber to formulate breads; CS as a food ingredient reduces caloric density and increases the dietary fiber content of bread (Pourfarzad et al., 2013). CS has also been used as a coloriant and as a dietary fiber source, to achieve a healthier, nutritious and a high sensorial quality biscuit. CS improved moisture, texture, thickness and color of the novel biscuits (Garcia-Serna, Martinez-Saez, Mesias, Morales, & Castillo, 2014). CS has also been used in another bakery product, cakes have been formulated with up to 30 % of water-treated CS as a flour substitute (Ateş & Elmacı, 2018b, 2018a). Water treatment of CS enhanced moisture content, textural and sensory attributes of cakes. Cakes with water treated CS presented similar physical and sensory characteristics to the control cake (Ateş & Elmacı, 2018b). The combined use of stevia and CS has also been used to achieve healthier, nutritious and good quality biscuits (Garcia-Serna et al., 2014). The complete replacement of sucrose by stevia affected the moisture content of the biscuits, but this was improved by the addition of CS. The nutritional value and the appearance of the biscuits also improved by the addition of CS (Garcia-Serna et al., 2014).

SCGs can be also be considered a source of proteins and, as the rest of coffee by-products, SCGs are high in fiber too (Table 2). Their nutritional value makes them interesting for their application in the manufacture of pastry and confectionery foods such as bread, cookies, and breakfast cereals, among others, resulting in a simple, low-cost method (PCT/ES2014/070062, 2014). This by-product has been employed in diverse combinations with other innovative ingredients, such as a hypocaloric sweetener (stevia) and soluble fiber (FOS) (Martinez-Saez, Tamargo García, et al., 2017). SCGs (4 % w/w) can be used directly as a food ingredient in solid foods such as cookies without affecting conventional food preparation and the final quality of the product. The application of SCGs represents a value-added opportunity for coffee by-product utilization at a very low cost.

3.3. Health promoting properties

Since more than 95 % of chronic diseases are caused by food choices and lack of physical exercise, many plant extracts and natural compounds are emerging as functional candidates to reduce the risk of non-communicable chronic diseases. The recycling of food wastes into health promoting products is of great interest worldwide (Kumar et al., 2017). In particular, the coffee industry is responsible for generating large amounts of wastes and coffee by-products may be sustainable sources of bioactive compounds with health promoting and therapeutic properties.

Coffee husks are enriched in anthocyanins, which contain powerful inhibitors of α-glucosidase and α-amylase enzymes. As these enzymes play an important role in the management of glucose metabolism, the use of anthocyanin extracts from coffee husks has been proposed as an antidiabetic agent to improve postprandial blood glucose metabolism (Murthy et al., 2012). These anti-diabetic properties have been also studied in a PhD thesis recently defended in our research group in which coffee husks were used as food ingredients for the development of innovative and sustainable functional yogurts. The yogurt containing coffee husks showed a high sensory acceptance (6.96), high antioxidant capacity (5.03 ± 0.11 µTE/g yogurt), inhibited α-glucosidase activity (83 %) *in vitro* and showed anti-inflammatory properties in a cellular model (Iriondo-DeHond, 2019). In addition, the digest obtained from the *in vitro* simulated human digestion of coffee husks yogurt showed antioxidant capacity and still inhibited α-glucosidase.

A recent study concerning the anti-inflammatory properties of coffee husks obtained from a semi-dry processing (Magoni et al., 2018) showed that they were capable of inhibiting the release of the pro-inflammatory cytokine IL-8 on gastric epithelial cells when inflammation was induced by TNF-α (Magoni et al., 2018). Caffeine present in coffee husks may also be one of the contributors to the overall anti-inflammatory effect observed in the by-product (J. H. Hwang, Kim, Ryu, & Lee, 2016; S. J. Hwang, Kim, Park, Lee, & Kim, 2014).

Nowadays, tea made from coffee husks has become a popular beverage commercialized by different companies all over the world. This beverage is known as "Cascara Tea" and it is sold as a super food with polyphenols with antioxidant properties and with Brain Derived Neurotrophic Factor (BDNF) that contributes to maintaining brain's health (Secret Cherry Tea, 2019).

The coffee mucilage honey mentioned in the previous section, is sold as a concentrate of antioxidants with health promoting properties such as strengthening the immune system (Natucafé S.A.S., 2019). With regard to the next by-product generated during coffee processing, coffee parchment, this by-product presented *in vitro* hypoglycemic properties, showing high glucose adsorption capacity (50–200 mmol/L) and α-amylase inhibition (52%); and also, this by-product showed *in vitro* hypolipidemic properties by pancreatic lipase inhibition (43%) and cholesterol binding (16.6 mg/g) (Benitez et al., 2019).

The high dietary fiber (up to 55 %) content of CS, predominantly insoluble fiber, can potentially benefit the intestine and gut microbiota (Ballesteros et al., 2014; A. S. G. Costa et al., 2018; Jiménez-Zamora et al., 2015). The prebiotic properties of CS

demonstrate that it preferentially supports the bifidobacteria growth *in vitro*, suggesting that its consumption may have some prebiotic effects (Borrelli et al., 2004).

The anti-obesity effect (reduction of the body fat mass and body fat percentage) of coffee may be attributed to caffeine (Kobayashi-Hattori, Mogi, Matsumoto, & Takita, 2005), chlorogenic acid (Cho et al., 2010) and melanoidins that are also present in CS (Mesías & Delgado-Andrade, 2017). Anti-obesity properties of CS have been studied in novel antioxidant beverages based on CS from Arabica and Robusta species to determine their inhibitory effect on *in vivo* fat accumulation using *Caenorhabditis elegans* as an animal model (Martinez-Saez et al., 2014). Both beverages, reduced body fat by 21 % and 24 %, respectively, possibly due to the presence of these compounds at physiologically active doses (Martinez-Saez et al., 2014). Furthermore, Robusta CSE beverage and a commercial dietary supplement that is made from Robusta decaffeinated green coffee extract showed a similar effect on body-fat reduction. Phenolic compounds composing aqueous extracts from coffee husks and CS also alleviated the complications of adipogenesis and inflammation *in vitro* (Rebollo-Hernanz, Zhang, Aguilera, Martín-Cabrejas, & Gonzalez de Mejia, 2019).

In an *in vivo* study with Wistar rats, an aqueous extract from CS (CSE) reduced total cholesterol and triglycerides plasma levels in rats after 45 days treatment with CSE (2.2 and 0.8 mg caffeine and CGA /kg body weight). Furthermore, CSE (36 mg/mL) reduced (41.73 %) pancreatic lipase activity *in vitro* (WO/2016/097450, 2016). This could explain the mechanism of action of the observed reduction of total cholesterol and triglycerides since pancreatic lipase is a key enzyme in fat digestion. Together, these results support the lyporegulatory character of CS through pancreatic lipase inhibition and therefore its preventive and therapeutic effect in obesity.

The components present in CSE have a positive effect on pancreas health, thereby reducing the risk of diabetes. Several studies have described the effects of CS on diabetes biomarkers. This by-product obtained from coffee roasting has been shown to produce increased glucose tolerance (WO/2016/097450, 2016), enhance insulin sensitivity and secretion (WO/2016/097450, 2016; Fernandez-Gomez, Ramos, et al., 2016), inhibit enzymatic α-glucosidase activity (Fernandez-Gomez, Ramos, et al., 2016), inhibit Advanced Glycation End Product (AGEs) formation through the interaction of CGA and its derivatives with protein backbone (Fernández-Gómez, 2016; Fernandez-Gomez et al., 2015) and protect against oxidative stress (K., L., & V.P., 2010). Moreover, CSE may protect pancreatic tissue *in vitro* against oxidative stress induced by the commonly used diabetogenic agent streptozotocin (STZ) (Maria D. del Castillo et al., 2017). All

these effects have an impact on diabetes development and, consequently, CSE may be useful in both the prevention and treatment of diabetes.

The connection between nutrition, skin and aging has been an increasing research area for scientists and physicians worldwide (Pearson, 2015). CS is a potential candidate to replace synthetic chemicals as active ingredients in cosmetic formulations due to its high antioxidant potential, phenolic compounds, melanoidins and caffeine content (Bessada, Alves, Oliveira, & Applications, 2018). The interest of using CS aqueous extract in cosmetics was proposed for the first time by del Castillo *et al*., in a patent application filed in 2011, which became public in 2013 (WO/2013/004873). Recent research demonstrates the hyaluronidase inhibitory activity of CS extracts presumably due to acidic polysaccharides mainly composed by uronic acid (Furusawa, Narita, Iwai, Fukunaga, & Nakagiri, 2011). Hyaluronidase degrades hyaluronic acid, reducing its viscosity, increasing permeability and leading to extracellular matrix (collagen and elastin fibers) destruction (Sahasrabudhe & Deodhar, 2010). The hyaluronidase inhibitory effect of CS has also been studied *in vivo*. Administration of a CS-based cream twice daily improved skin hydration and firmness of 20 human volunteers after 28 days. CS was an effective emollient ingredient, with results similar to hyaluronic acid (Francisca Rodrigues, Matias, Ferreira, Amaral, & Oliveira, 2016).

The by-products generated in the roasting and brewing process, CS and SCGs respectively, are the most studied regarding their nutritional value and health promoting properties (Table 2). SCGs can be directly employed as a sustainable natural source of dietary fiber (PCT/ES2014/070062, 2014). However, fiber can be also extracted from the raw material employing different processes, such as ohmic technology (MX/a/2016008578, 2016; Vazquez Sanchez, Martinez-Saez, del Castillo, & Campos-Vega, 2015), alkaline hydrogen peroxide treatment (Vilela, Leão, Franca, & Oliveira, 2016), and autohydrolysis (Ballesteros, Teixeira, & Mussatto, 2017). SCGs dietary fiber can be fermented by colon microbiota, producing short-chain fatty acids (SCFAs) with anti-inflammatory properties, such as inhibition of nitric oxide production and other inflammatory mediators such as IL-10, CCL-17, CXCL9, IL-1β and IL-5 cytokines (López-Barrera, Vázquez-Sánchez, Loarca-Piña, & Campos-Vega, 2016). Consequently, SCGs have been proposed as a protective agent against chronic inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis. In addition, dietary fiber from SCGs has recently been reported to stimulate the release of the satiety hormone, serotonin, and glucagon-like peptide-1 (Martinez-Saez, Hochkogler, Somoza, & del Castillo, 2017). The antioxidant properties of the dietary fiber of SCGs have mainly been ascribed to the presence of phenolic compounds present

in their polymeric structure (Martinez-Saez, Tamargo García, et al., 2017). Furthermore, SCGs exert a positive effect on beneficial bacteria, increasing the numbers of lactobacilli and bifidobacteria, being a good source of prebiotic compounds (Jiménez-Zamora et al., 2015).

3.4. Safety

The information regarding the analysis of substances of toxicological concern should be included in the application for authorization of a novel food in the context of the Regulation (EU) 2015/2283. First, information on the identities and quantities of impurities, residues and chemical and microbiological contaminants should be provided in the compositional data section (e.g. heavy metals, mycotoxins, PCBs/dioxins, pesticides) when applying for a novel food (Figure 2) (EFSA Panel on Dietetic Products, 2016).

After analysis of substances of possible concern to human health, toxicological studies should be carried out with the novel food. First, studies at a genomic level must be carried out since the assessment of genotoxic potential is a basic component of chemical risk assessment. Then, in addition to an acute toxicity assay, a subchronic toxicity study should normally be submitted to identify any adverse effects following prolonged exposure via an appropriate oral route. Moreover, reproductive and developmental toxicity tests may be required if there are any indications of effects on reproductive organs or parameters. Ultimately, human studies, if available, should be provided if they contain information relevant for the safety assessment, such as physical examination, blood chemistry, hematology, urine analysis, blood pressure and organ function tests and/or monitoring of adverse reactions (EFSA Panel on Dietetic Products, 2016).

Limitations on the use of coffee by-products are connected to its content in caffeine. Coffee by-products present different levels of caffeine. However, in all cases their content is lower than that found in green and roasted coffee beans (Farah, 2012). Although further studies should be conducted in this field, so far published results suggest caffeine content does not need to be considered as a safety concern in the application of coffee by-products as food ingredients. The caffeine content in foods formulated with coffee by-products should be below the European Food Safety Authority (EFSA) safety level for daily caffeine consumption of 400 mg for the general population and 200 mg for lactating women. For children and adolescents, the available information is insufficient to derive a safe level of caffeine intake (European Food Safety

Authority (EFSA), 2015). Therefore, no limitations on the use of these by-products as food ingredients for human nutrition need to be considered.

All foods can contain chemical and/or biological contaminants. Another limitation on the use of coffee by-products obtained after the roasting process, CS and SCGs, is acrylamide, a chemical-processing contaminant. Acrylamide is formed during the roasting of coffee beans through the Maillard reaction, during which the aroma and the color of coffee beans are also produced. This reaction takes place when precursors, for example, reducing sugars, such as glucose and fructose, and asparagine, are present in raw materials, in combination with a high temperature and long cooking time (Pedreschi, Mariotti, & Granby, 2014). Low levels of 11.42 µg/L and 37 µg/kg of acrylamide have been previously found in CS and SCGs, respectively (Garcia-Serna et al., 2014; Martinez-Saez, Tamargo García, et al., 2017). These values are under the limit established by the EFSA for coffee and instant coffee, 450 and 900 µg/kg, respectively (European Food Safety Authority, 2015).

Ochratoxin A (OTA) is a biological contaminant, a mycotoxin produced by *Aspergillus ochraceus* and *Penicillium verrucosum* that tends to bioaccumulate along the food chain. This mycotoxin can induce renal toxicity, nephropathy, and immunosuppression, representing a risk to human safety (Toschi et al., 2014). Coffee is considered a secondary source of OTA in the human diet. OTA is already present in coffee before storage, as contamination can occur due to several climatic conditions, coffee fruits falling onto the soil, transportation, and so on. Therefore, the critical steps leading to the accumulation of this mycotoxin are the harvesting and the postharvest handling of coffee cherries (Napolitano et al., 2007). Reported values of OTA in CS were of 18.7 − 34.4 µg/kg, which is about 3 times higher than the European Commission limits for coffee products (5 µg/kg) (Toschi et al., 2014). However, Ferraz *et al*. (2010) demonstrated that OTA can be destroyed during roasting (Ferraz et al., 2010). Even when the coffee beverage is prepared from highly contaminated green beans, the coffee-transforming process is able to reduce the amount of OTA that presents a risk to human health (Napolitano et al., 2007). Nevertheless, as in other food ingredients, it is very important to establish rigorous quality controls along the coffee-bean processing chain to reduce OTA and ensure the healthiness of coffee and its by-products (Napolitano et al., 2007).

In addition to the determination of biological, chemical and processing contaminants, safety of coffee by-products needs to be further confirmed by toxicity studies. To the best of our knowledge, no toxicity studies of coffee by-products have been carried out. However, CoffeeBerry® products (a powdered extract, a water extract and a water–

ethanol extract) are derived from the whole fruit their safety has been evaluated in genotoxicity studies, short-term oral toxicity studies and a 90-day dietary toxicity study (Heimbach et al., 2010). None of the coffee cherry extracts showed mutagenic or genotoxic potential, and in short-term and 90 days toxicity studies, no adverse effects at the studied concentrations were observed (Heimbach et al., 2010).

One study has been published regarding the skin compatibility and safety of CS extracts for topical use *in vitro* using a human skin model and *in vivo* by patch tests. Results from *in vitro* and *in vivo* studies revealed that CS is safe regarding skin irritancy (F. Rodrigues et al., 2015). *In vitro* studies revealed that the body cream formulation containing CS extract is safe in contact with human skin cells (fibroblasts and keratinocytes) and the patch test carried out with this formulation proved the absence of skin irritation (F Rodrigues, Sarmento, Amaral, & Oliveira, 2016).

Further determination of pesticides and mycotoxins in coffee by-products are needed to ensure their safety for human consumption. In addition, toxicological studies at different levels (genotoxicity, acute toxicity and repeated dose toxicity) are needed for the validation of coffee by-products as novel food ingredients.

3.5. Regulatory status

The main consideration regarding the use of coffee by-products as food ingredients is their regulatory status. In December 2016 the European Food Safety Authority (EFSA) included the coffee cherry in the Novel Food Catalog (Figure 11) (European Comission, 2019). Therefore, coffee husks needed to undergo EFSA's authorization procedure according to the Regulation (EU) 2015/2283 before it could be introduced into the European food market (Figure 2).

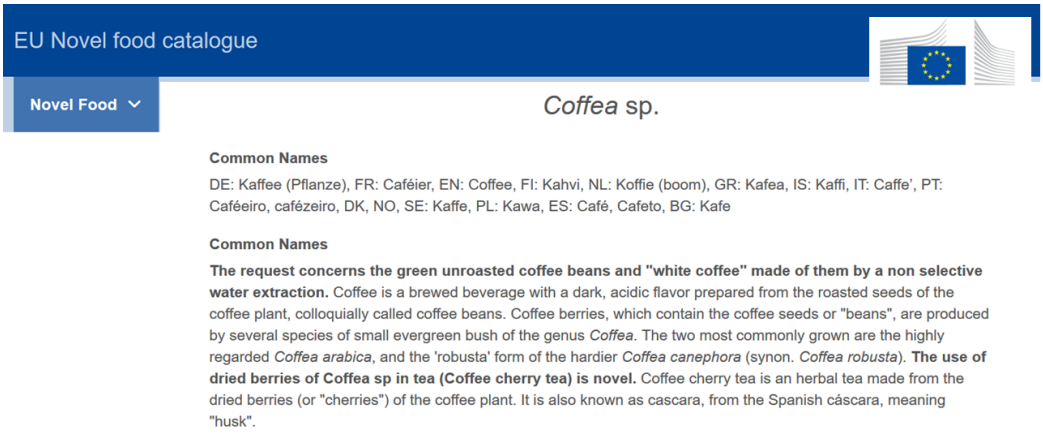


Figure 11. Coffee cherry in the EU Novel food catalogue of the European Commission requires following the Regulation (EU) 2015/2283 for its authorization as a novel ingredient.

The application for the authorization of husk as a novel food in order to be approved for consumption by the European Commission in the European Union has been submitted by Joel Jelderks, CEO and Founder of Caskai, a leading brand of coffee husks based beverages. To date, coffee husks are waiting for its approval by the Committee to be introduced in the union list of authorized novel foods. Meanwhile, coffee coffee husks an officially authorized ingredient in other parts of the world, such as Mexico or the United States (US), where it is generally recognized as safe (GRAS).

Novel ingredients obtained from other coffee by-products, such as mucilage, parchment and silverskin, should also undergo EFSA's authorization procedure according to the Regulation (EU) 2015/2283. With regard to spent coffee grounds, the application for authorizing powder from SCGs has been submitted within the meaning of Article 10(1) of Regulation (EU) 2105/2283 by Kaffe Bueno ApS, Denmark. In addition, the whole roasted coffee bean is consumed in other countries, such as US, as a chocolate-covered candy and it is included in the National Nutrient Database of the US Department of Agriculture (USDA). Therefore, it can be said that the insoluble fraction obtained after coffee brewing, SCGs, which is present in the coffee bean has a history of use in other countries.

Coffee by-products, especially CS and SCGs that are the most studied, have not been industrially exploited and there are not commercially available products containing these potential novel ingredients. It is of great importance that coffee industries make an effort to valorize by-products that result from coffee processing in order to increase the

sustainability of the process, increase economical incomes and create new jobs in producing countries (Janissen & Huynh, 2018). The total recycling of the cherry into health-promoting foods and beverages with particular nutrition and health claims may contribute to a sustainable nutrition and health of the population. This investigation aims to contribute to this challenge.

4. References

Acevedo, F., Rubilar, M., Scheuermann, E., Cancino, B., Uquiche, E., Garcés, M., … Shene, C. (2013). Spent coffee grounds as a renewable source of bioactive compounds. *Journal of Biobased Materials and Bioenergy*, *7*(3), 420–428. https://doi.org/10.1166/jbmb.2013.1369

Aguilera, Y., Rebollo-Hernanz, M., Cañas, S., Taladrid, D., & Martín-Cabrejas, M. A. (2019). Response surface methodology to optimise the heat-assisted aqueous extraction of phenolic compounds from coffee parchment and their comprehensive analysis. *Food & Function*, (C). https://doi.org/10.1039/c9fo00544g

Alghooneh, A., Mohammad Amini, A., Behrouzian, F., & Razavi, S. M. A. (2017). Characterisation of cellulose from coffee silverskin. *International Journal of Food Properties*, *20*(11), 2830–2843. https://doi.org/10.1080/10942912.2016.1253097

Alves, R. C., Rodrigues, F., Nunes, M. A. A., Vinha, A. F., & Oliveira, M. B. P. P. (2017). State of the art in coffee processing by-products. In C. Galanakis (Ed.), *Handbook of Coffee Processing By-Products: Sustainable Applications* (pp. 1–26). Academic Press-Elsevier.

Andrade, K. S., Gonçalvez, R. T., Maraschin, M., Ribeiro-do-Valle, R. M., Martínez, J., & Ferreira, S. R. S. (2012). Supercritical fluid extraction from spent coffee grounds and coffee husks: antioxidant activity and effect of operational variables on extract composition. *Talanta*, *88*, 544–552. https://doi.org/10.1016/j.talanta.2011.11.031

Apuzzo, J. (2017). Cellulosic composition containing coffee parchment cellulose and uses thereof.

Arulrajah, A., Kua, T.-A., Horpibulsuk, S., Mirzababaei, M., & Chinkulkijniwat, A. (2017). Recycled glass as a supplementary filler material in spent coffee grounds geopolymers. *Construction and Building Materials*, *151*, 18–27. https://doi.org/10.1016/J.CONBUILDMAT.2017.06.050

Ateş, G., & Elmacı, Y. (2018a). Coffee silverskin as fat replacer in cake formulations and its effect on physical, chemical and sensory attributes of cakes. *Lwt*, *90*, 519–525. https://doi.org/10.1016/j.lwt.2018.01.003

Ateş, G., & Elmacı, Y. (2018b). Physical, chemical and sensory characteristics of fiber-enriched cakes prepared with coffee silverskin as wheat flour substitution. *Journal of Food Measurement and Characterization*, *0*(0), 0. https://doi.org/doi.org/10.1007s11694-018-9988-9

Avallone, S., Guiraud, J.-P. J.-P., Guyot, B., Olguin, E., & Brillouet, J.-M. J.-M. (2000). Polysaccharide Constituents of Coffee-Bean Mucilage. *Journal of Food Science*, *65*(8), 1308–1311. https://doi.org/10.1111/j.1365-2621.2000.tb10602.x

Baiano, A. (2014). Recovery of biomolecules from food wastes - A review. *Molecules*, *19*(9), 14821–14842. https://doi.org/10.3390/molecules190914821

Ballesteros, L. F., Teixeira, J. a., & Mussatto, S. I. (2014). Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. *Food and Bioprocess Technology*, *7*(12), 3493–3503. https://doi.org/10.1007/s11947-014-1349-z

Ballesteros, L. F., Teixeira, J. A., & Mussatto, S. I. (2017). Extraction of polysaccharides by autohydrolysis of spent coffee grounds and evaluation of their antioxidant activity. *Carbohydrate Polymers*, *157*, 258–266. https://doi.org/10.1016/j.carbpol.2016.09.054

Barczynska, R., Bandurska, K., Slizewska, K., Litwin, M., Szalecki, M., Libudzisz, Z., & Kapusniak, J. (2015). Intestinal microbiota, obesity and prebiotics. *Polish Journal of Microbiology*, *64*(2), 93–100.

Barrett, A. (2019). Cellulose Film Packaging Gains Popularity with Plastic Reduction Initiatives. Retrieved from https://bioplasticsnews.com/2019/02/22/cellulose-film-packaging-gains-popularity-with-plastic-reduction-initiatives/

Bekalo, S. A., & Reinhardt, H.-W. W. (2010). Fibers of coffee husk and hulls for the production of particleboard. *Materials and Structures*, *43*(8), 1049–1060. https://doi.org/DOI 10.1617/s11527-009-9565-0

Belitz, H.-D., Grosch, W., & Schieberle, P. (2009). Coffee, tea, cocoa. In *In: Food Chemistry. Eds. Belitz, H.-D., Grosch, W. and Schieberle, P., 4th edition, Springer, Leipzig* (pp. 938–951).

Benitez, V., Rebollo-Hernanz, M., Hernanz, S., Chantres, S., Aguilera, Y., & Martin-Cabrejas, M. A. (2019). Coffee parchment as a new dietary fiber ingredient: Functional and physiological characterization. *Food Research International*, *122*(December 2018), 105–113. https://doi.org/10.1016/j.foodres.2019.04.002

Bessada, S. M. F., Alves, R. C., Costa, A. S. G., Nunes, M. A., & Oliveira, M. B. P. P. (2018). Coffea canephora silverskin from different geographical origins: A comparative study. *Science of the Total Environment*, *645*, 1021–1028. https://doi.org/10.1016/j.scitotenv.2018.07.201

Bessada, S. M. F., Alves, R. C., Oliveira, M. B. P. P., & Applications, C. (2018). Coffee Silverskin : A Review on Potential Cosmetic Applications. *Cosmetics*, *5*(1), 5. https://doi.org/10.3390/cosmetics5010005

Bio-bean. (2013). Coffee Logs. Retrieved from https://www.bio-bean.com/coffee-logs/#hero

Borrelli, R. C., Esposito, F., Napolitano, A., Ritieni, A., & Fogliano, V. (2004). Characterization of a new potential functional ingredient: coffee silverskin. *Journal of Agricultural and Food Chemistry*, *52*(5), 1338–1343. https://doi.org/10.1021/jf034974x

Brand, D., Pandey, a., Rodriguez-Leon, J. a., Roussos, S., Brand, I., & Soccol, C. R. (2001). Packed bed column fermenter and kinetic modeling for upgrading the nutritional quality of

coffee husk in solid-state fermentation. *Biotechnology Progress*, *17*(6), 1065–1070. https://doi.org/10.1021/bp010112+

Bravo, J., Jua, I., Monente, C., Caemmerer, B., & Kroh, L. W. (2012). Evaluation of Spent Co ff ee Obtained from the Most Common Co ff eemakers as a Source of Hydrophilic Bioactive Compounds.

Bresciani, L., Calani, L., Bruni, R., Brighenti, F., & Del Rio, D. (2014). Phenolic composition, caffeine content and antioxidant capacity of coffee silverskin. *Food Research International*, *61*, 196–201. https://doi.org/10.1016/j.foodres.2013.10.047

Campos-Vega, R., Vázquez-Sánchez, K., López-Barrera, D., Loarca-Piña, G., Mendoza-Díaz, S., & Oomah, B. D. (2015). Simulated gastrointestinal digestion and in vitro colonic fermentation of spent coffee (Coffea arabica L.): Bioaccessibility and intestinal permeability. *Food Research International*, *77*, 156–161. https://doi.org/10.1016/j.foodres.2015.07.024

Campos-Vega, Rocio, Gaytan Martinez, M., Vazquez Sanchez, K., & Morales Sanchez, E. (2016). *MX/a/2016008578*.

Campos-Vega, Rocio, Loarca-Piña, G., Vergara-Castañeda, H., & Oomah, B. D. (2015). Spent coffee grounds: A review on current research and future prospects. *Trends in Food Science & Technology*, *45*(1), 24–36. https://doi.org/10.1016/j.tifs.2015.04.012

Chen, J. (2009). S.Café®. Retrieved from http://www.scafefabrics.com/en-global/home/index

Cherry, P. (1974). Method for drying moisture from wet spent coffee grounds.

Cho, A. S., Jeon, S. M., Kim, M. J., Yeo, J., Seo, K. Il, Choi, M. S., & Lee, M. K. (2010). Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. *Food and Chemical Toxicology*, *48*(3), 937–943. https://doi.org/10.1016/j.fct.2010.01.003

Corrêa, J. L. G., Santos, J. C. P., Fonseca, B. E., & Carvalho, A. G. da S. (2014). Drying of spent coffee grounds in a cyclonic dryer. *Coffee Science*, *9*(1), 68–76. https://doi.org/10.25186/cs.v9i1.544

Costa, A. S. G., Alves, R. C., Vinha, A. F., Costa, E., Costa, C. S. G., Nunes, M. A., … Oliveira, M. B. P. P. (2018). Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product. *Food Chemistry*, *267*, 28–35. https://doi.org/10.1016/j.foodchem.2017.03.106

Costa, A. S. G. G., Alves, R. C., Vinha, A. F., Barreira, S. V. P. P., Nunes, M. a., Cunha, L. M., & Oliveira, M. B. P. P. P. (2014). Optimization of antioxidants extraction from coffee silverskin, a roasting by-product, having in view a sustainable process. *Industrial Crops and Products*, *53*, 350–357. https://doi.org/10.1016/j.indcrop.2014.01.006

Cubero-Castillo, E., Bonilla-Leiva, A. R., & García-Velasques, E. (2017). Coffee Berry Processing By-Product Valorization : Coffee Parchment as a Potential Fiber Source to Enrich Bakery Goods Abstract. *Journal of Food, Nutrition and Population Health*, *1*(2), 1–7.

De Bruyn, F., Zhang, S. J., Pothakos, V., Torres, J., Lambot, C., Moroni, A. V., … De Vuysta,

L. (2017). Exploring the Impacts of Postharvest Processing on the Microbiota and Metabolite Profiles during Green Coffee Bean Production. *Applied and Environmental Microbiology*, *83*(1), 1–16.

del Castillo, M. Dolores, Fernandez-Gomez, B., Martinez-Saez, N., Iriondo-DeHond, A., & Mesa, M. D. (2019). Coffee By-Products. In A. Farah (Ed.), *Coffee: Production, Quality and Chemistry*. Oxfordshire: Royal Society of Chemistry.

del Castillo, Maria D., Iriondo-DeHond, A., Martinez-Saez, N., Fernandez-Gomez, B., Iriondo-DeHond, M., & Zhou, J.-R. (2017). Chapter 6 – Applications of recovered compounds in food products. In *Handbook of Coffee Processing By-Products* (pp. 171–194). https://doi.org/10.1016/B978-0-12-811290-8.00006-2

del Castillo, Maria Dolores, Fernandez-Gomez, B., Ullate, M., Mesa, M. D., & Del Castillo, M.D., Fernandez-Gomez, B., Ullate, M. and Mesa, M. D. (2016). *WO/2016/097450*. Oficina Española de Patentes y Marcas. Retrieved from https://patentscope.wipo.int/search/es/detail.jsf?docId=WO2016097450

del Castillo, María Dolores, Ibañez, M. E., Amigo, M., Herrero, M., Plaza del Moral, M., & Ullate, M. (2013). Application of products of coffee silverskin in anti-ageing cosmetics and functional food. WO 2013/004873.

del Castillo, María Dolores, Iriondo-DeHond, A., & Martirosyan, D. M. (2018). Are functional foods essential for sustainable health? *Annals of Nutrition & Food Science*.

del Castillo, María Dolores, Martinez-Saez, N., & Ullate, M. (2014). *PCT/ES2014/070062*. Spain.

Delzenne, N. M., Cani, P. D., Daubioul, C., & Neyrinck, A. M. (2005). Impact of inulin and oligofructose on gastrointestinal peptides. *British Journal of Nutrition*, *93*(S1), S157. https://doi.org/10.1079/BJN20041342

Demir, M. K., & Elgün, A. (2014). Comparison of autoclave, microwave, IR and UV-C stabilization of whole wheat flour branny fractions upon the nutritional properties of whole wheat bread. *Journal of Food Science and Technology*, *51*(1), 59–66. https://doi.org/10.1007/s13197-011-0475-0

Deora, N. S. (2018). Whole wheat flour stability: An insight. *Acta Scientific Nutritioal Health*, *2*(3), 8–18.

Diaz, V. C. (2019). Know the Novel Foods. Retrieved from https://knowmadinstitut.org/2019/05/know-the-novel-foods/

Du, H., van der A, D. L., Boshuizen, H. C., Forouhi, N. G., Wareham, N. J., Halkjær, J., … Feskens, E. J. (2010). Dietary fiber and subsequent changes in body weight and waist circumference in European men and women. *The American Journal of Clinical Nutrition*, *91*(2), 329–336. https://doi.org/10.3945/ajcn.2009.28191

EFSA Panel on Dietetic Products. (2016). Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. *EFSA Journal Journal*, *14*(September), 11. https://doi.org/10.2903/j.efsa.2016.NNNN

Esquivel, P., & Jiménez, V. M. (2012). Functional properties of coffee and coffee by-products.

Food Research International, *46*(2), 488–495. https://doi.org/10.1016/j.foodres.2011.05.028

European Comission. (2019). Novel food catalogue.

European Commission. (2005). COMMISSION REGULATION (EC) No 123/2005 of 26 January 2005 amending Regulation (EC) No 466/2001 as regards ochratoxin A. *Official Journal of the European Union*, *2005*(123), 2004–2006. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R0123&qid=1478028660364&from=ES

European Commission. (2012). COMMISSION REGULATION (EU) No 1047/2012. *Official Journal of the European Union*, *2012*(1047), 36–37.

European Commission. (2017). COMMISSION REGULATION (EU) 2017/2158 of 20 November 2017 establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food. *Official Journal of the European Union*, *204*, 24–44. https://doi.org/http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_285/l_28520031101en00330037.pdf

European Commission. (2018). Bioeconomy, FOOD 2030. Retrieved from https://ec.europa.eu/research/bioeconomy/index.cfm?pg=policy&lib=food2030

European Council. (1990). Council Directive of 24 September 1990 on nutrition labelling for foodstuffs (90/496/ EEC). *Official Journal of the European Union*, (L), 18–63. Retrieved from http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32011R1169

European Food Safety Authority. (2015). Scientific Opinion on acrylamide in food. *EFSA Journal*, *13*(6), 4104. https://doi.org/10.2903/j.efsa.2015.4104

European Food Safety Authority (EFSA). (2010). Scientific Opinion on Dietary Reference Values for carbohydrates and dietary fibre. *EFSA Journal*, *8*(3), 1–77. https://doi.org/10.2903/j.efsa.2010.1462.Available

European Food Safety Authority (EFSA). (2015). Scientific Opinion on the safety of caffeine. *EFSA Journal*, *13*(5), 1–21. https://doi.org/10.2903/j.efsa.2015.4102

Fahim, I. S., Chbib, H., & Mahmoud, H. M. (2019). The synthesis, production & economic feasibility of manufacturing PLA from agricultural waste. *Sustainable Chemistry and Pharmacy*, *12*(March), 100142. https://doi.org/10.1016/j.scp.2019.100142

Fairweather, A. (n.d.). Green Cup. Retrieved from http://greencup.co.uk/recycling-upcycling/

FAO, & Food Climate Research Network. (2016). *Plates, pyramids, planet*. https://doi.org/ISBN 978-92-5-109222-4

Farah, A. (2012). Coffee Constituents. *Coffee: Emerging Health Effects and Disease Prevention*, 21–58. https://doi.org/10.1002/9781119949893.ch2

Fernández-Gómez, B. (2016). Sustainable use of coffee silverskin as a natural source of bioactive compounds for diabetes, (April), 87–132.

Fernandez-Gomez, B., Lezama, A., Amigo-Benavent, M., Ullate, M. M., Herrero, M., Martín,

M. Á., … del Castillo, M. D. M. D. (2016). Insights on the health benefits of the bioactive compounds of coffee silverskin extract. *Journal of Functional Foods*, *25*(6), 197–207. https://doi.org/10.1016/j.jff.2016.06.001

Fernandez-Gomez, B., Ramos, S., Goya, L., Mesa, M. D., del Castillo, M. D., & Martín, M. Á. (2016). Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells. *Food Research International*. https://doi.org/.doi.org/10.1016/j.foodres.2016.03.006

Fernandez-Gomez, B., Ullate, M., Picariello, G., Ferranti, P., Mesa, M. D., & del Castillo, M. D. (2015). New knowledge on the antiglycoxidative mechanism of chlorogenic acid. *Food Funct.*, *6*(6), 2081–2090. https://doi.org/10.1039/C5FO00194C

Fernandez-Vasallo, C. (2017). *Harinas especiales*. *XXIX AETC Jornadas Técnicas*. Valladolid. Retrieved from http://www.aetc.es/wp-content/uploads/2017/11/Harinas-Especiales.-Carlos-Fernández-Vasallo.-MOLENDUM-1.pdf

Ferrão, J. E. M. (2009). *O Café, A Bebida Negra Dos Sonhos Claros*. Lisboa, Portugal.: Chaves Ferreira-Publicações S. A.

Ferraz, M. B. M., Farah, A., Iamanaka, B. T., Perrone, D., Copetti, M. V., Marques, V. X., … Taniwaki, M. H. (2010). Kinetics of ochratoxin A destruction during coffee roasting. *Food Control*, *21*(6), 872–877. https://doi.org/10.1016/j.foodcont.2009.12.001

Food and Agriculture Organization of the United Nations (FAO). (2011). *Global food losses and food waste – Extent, causes and prevention*. Rome. https://doi.org/10.1098/rstb.2010.0126

Food and Agriculture Organization of the United Nations (FAO). (2018). Sustainable Bioeconomy Guidelines. Retrieved from http://www.fao.org/energy/bioeconomy/en/

Food and Agriculture Organization of the United Nations (FAO). (2019). Development of a Code of Conduct on Food Loss and Food Waste Prevention. Retrieved from http://www.fao.org/fsnforum/es/node/4877

Furusawa, M., Narita, Y., Iwai, K., Fukunaga, T., & Nakagiri, O. (2011). Inhibitory effect of a hot water extract of coffee "silverskin" on hyaluronidase. *Bioscience, Biotechnology, and Biochemistry*, *75*(6), 1205–1207. https://doi.org/10.1271/bbb.110106

FUTURE CEUTICALS. (1999). Retrieved from https://www.futureceuticals.com/

Garcia-Serna, E., Martinez-Saez, N., Mesias, M., Morales, F. J., & Castillo, M. D. Del. (2014). Use of Coffee Silverskin and Stevia to Improve the Formulation of Biscuits. *Polish Journal of Food and Nutrition Sciences*, *64*(4), 243–251. https://doi.org/10.2478/pjfns-2013-0024

Gómez-De La Cruz, F. J., Cruz-Peragón, F., Casanova-Peláez, P. J., & Palomar-Carnicero, J. M. (2015). A vital stage in the large-scale production of biofuels from spent coffee grounds: The drying kinetics. *Fuel Processing Technology*, *130*(C), 188–196. https://doi.org/10.1016/j.fuproc.2014.10.012

Gonzalez, B. (2016). *Uso sostenible del pergamino de café como ingrediente alimentario en la elaboración de pan libre de gluten con alto contenido en fibra*. Universidad Autónoma de Madrid.

Gouvea, B. M., Torres, C., Franca, A. S., Oliveira, L. S., & Oliveira, E. S. (2009). Feasibility of ethanol production from coffee husks. *Biotechnology Letters*, *31*(9), 1315–1319. https://doi.org/10.1007/s10529-009-0023-4

Guglielmetti, A., Fernandez-Gomez, B., Zeppa, G., & del Castillo, M. D. (2019). Nutritional quality, potential health promoting properties and sensory perception of an improved gluten free bread formulation containing inulin, rice protein and bioactive compounds extracted from coffee byproducts. *Polish Journal of Food and Nutrition Sciences, Accepted*. https://doi.org/10.31883/pjfns-2019-0012

Hartati, I., Wahid, U., Riwayati, I., Wahid, U., Kurniasari, L., Wahid, U., & Hartati, I. (2012). Potential production of food colorant from coffee pulp, (December 2015).

Heeger, A., Kosińska-Cagnazzo, A., Cantergiani, E., & Andlauer, W. (2017). Bioactives of coffee cherry pulp and its utilisation for production of Cascara beverage. *Food Chemistry*, *221*, 969–975. https://doi.org/10.1016/j.foodchem.2016.11.067

Heimbach, J. T., Marone, P. A., Hunter, J. M., Nemzer, B. V., Stanley, S. M., & Kennepohl, E. (2010). Safety studies on products from whole coffee fruit. *Food and Chemical Toxicology*, *48*(8–9), 2517–2525. https://doi.org/10.1016/j.fct.2010.06.025

Hwang, J. H., Kim, K. J., Ryu, S. J., & Lee, B. Y. (2016). Caffeine prevents LPS-induced inflammatory responses in RAW264.7 cells and zebrafish. *Chemico-Biological Interactions*, *248*, 1–7. https://doi.org/10.1016/j.cbi.2016.01.020

Hwang, S. J., Kim, Y. W., Park, Y., Lee, H. J., & Kim, K. W. (2014). Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide- stimulated RAW 264.7 cells. *Inflammation Research*, *63*(1), 81–90. https://doi.org/10.1007/s00011-013-0674-4

Imbert, E. (2017). Food waste valorization options: opportunities from the bioeconomy. *Open Agriculture*, *2*(1), 195–204. https://doi.org/10.1515/opag-2017-0020

International Coffee Organization. (2019). *Total production by all exporting countries*. *Trade Statistics Tables*.

Iriondo-DeHond, M. (2019). *Development of novel functional dairy foods using byproducts from the coffee and wine industries for a sustainable nutrition and health*. Universidad Autonóma de Madrid.

Janissen, B., & Huynh, T. (2018). Chemical composition and value-adding applications of coffee industry by-products: A review. *Resources, Conservation and Recycling*, *128*(July 2017), 110–117. https://doi.org/10.1016/j.resconrec.2017.10.001

Jelderks, J., & Zimmermann, U. (2017). Caskai. Retrieved from https://caskai.com/

Jiménez-Zamora, A., Pastoriza, S., & Rufián-Henares, J. a. (2015). Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties. *LWT - Food Science and Technology*, *61*(1), 12–18. https://doi.org/10.1016/j.lwt.2014.11.031

K., K., L., P., & V.P., M. (2010). Combined treatment of tetrahydrocurcumin and chlorogenic acid exerts potential antihyperglycemic effect on streptozotocin-nicotinamide-induced diabetic rats. *General Physiology and Biophysics*, *29*(1), 23–30. https://doi.org/10.4149/gpb_2010_01_23

KAFFEE FORM. (2015). Retrieved September 19, 2019, from https://www.kaffeeform.com/de/

Kamal, I. M., Sobolik, V., Kristiawan, M., Mounir, S. M., & Allaf, K. (2008). Structure expansion of green coffee beans using instantaneous controlled pressure drop process. *Innovative Food Science and Emerging Technologies*, *9*(4), 534–541. https://doi.org/10.1016/j.ifset.2008.01.004

Kobayashi-Hattori, K., Mogi, A., Matsumoto, Y., & Takita, T. (2005). Effect of caffeine on the body fat and lipid metabolism of rats fed on a high-fat diet. *Biosci Biotechnol Biochem*, *69*(11), 2219–2223. https://doi.org/10.1271/bbb.69.2219

Kumar, K., Yadav, A. N., Kumar, V., Vyas, P., & Dhaliwal, H. S. (2017). Food waste: a potential bioresource for extraction of nutraceuticals and bioactive compounds. *Bioresources and Bioprocessing*, *4*(1), 18. https://doi.org/10.1186/s40643-017-0148-6

Limousy, L., Jeguirim, M., Labbe, S., Balay, F., & Fossard, E. (2015). Performance and emissions characteristics of compressed spent coffee ground/wood chip logs in a residential stove. *Energy for Sustainable Development*, *28*, 52–59. https://doi.org/10.1016/J.ESD.2015.07.002

López-Barrera, D. M., Vázquez-Sánchez, K., Loarca-Piña, M. G. F., & Campos-Vega, R. (2016). Spent coffee grounds, an innovative source of colonic fermentable compounds, inhibit inflammatory mediators in vitro. *Food Chemistry*, *212*, 282–290. https://doi.org/10.1016/j.foodchem.2016.05.175

Louis Bresson, J., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., Ildico Hirsch-Ernst, K., … Vinceti, M. (2016). SCIENTIFIC OPINION Draft guidance on the preparation and presentation of an application for authorisation of a Novel Food EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) Novel Food Guidance Panel members. *EFSA Journal Journal*. https://doi.org/10.2903/j.efsa.2016.NNNN

Magoni, C., Bruni, I., Guzzetti, L., Dell'Agli, M., Sangiovanni, E., Piazza, S., … Labra, M. (2018). Valorizing coffee pulp by-products as anti-inflammatory ingredient of food supplements acting on IL-8 release. *Food Research International*, *112*, 129–135. https://doi.org/10.1016/J.FOODRES.2018.06.026

Martinez-Saez, N., Hochkogler, C., Somoza, V., & del Castillo, M. (2017). Biscuits with No Added Sugar Containing Stevia, Coffee Fibre and Fructooligosaccharides Modifies α-Glucosidase Activity and the Release of GLP-1 from HuTu-80 Cells and Serotonin from Caco-2 Cells after In Vitro Digestion. *Nutrients*, *9*(7), 694. https://doi.org/10.3390/nu9070694

Martinez-Saez, N., Tamargo García, A., Domínguez Pérez, I., Rebollo-Hernanz, M., Mesías, M., Morales, F. J., … del Castillo, M. D. (2017). Use of spent coffee grounds as food ingredient in bakery products. *Food Chemistry*, *216*, 114–122. https://doi.org/10.1016/j.foodchem.2016.07.173

Martinez-Saez, N., Ullate, M., Martin-Cabrejas, M. a., Martorell, P., Genovés, S., Ramon, D., & Del Castillo, M. D. (2014). A novel antioxidant beverage for body weight control based on coffee silverskin. *Food Chemistry*, *150*, 227–234. https://doi.org/10.1016/j.foodchem.2013.10.100

Marto, J., Gouveia, L. F., Chiari, B. G., Paiva, A., Isaac, V., Pinto, P., … Ribeiro, H. M. (2016). The green generation of sunscreens: Using coffee industrial sub-products. *Industrial Crops and Products*, *80*, 93–100. https://doi.org/10.1016/J.INDCROP.2015.11.033

Massachusetts Institute of Technology. (2010). Ministry of Supply. Retrieved from https://ministryofsupply.com/

Mata, T. M., Martins, A. A., & Caetano, N. S. (2018). Bio-refinery approach for spent coffee grounds valorization. *Bioresource Technology*, *247*(July 2017), 1077–1084. https://doi.org/10.1016/j.biortech.2017.09.106

McMillan, R. (2014). ROSALIE MCMILLAN. Retrieved from http://www.rosaliemcmillan.com/

McNutt, J., & He, Q. (Sophia). (2019). Spent coffee grounds: A review on current utilization. *Journal of Industrial and Engineering Chemistry*, *71*, 78–88. https://doi.org/10.1016/j.jiec.2018.11.054

Meijer, K., De Vos, P., & Priebe, M. G. (2010). Butyrate and other short-chain fatty acids as modulators of immunity: What relevance for health? *Current Opinion in Clinical Nutrition and Metabolic Care*, *13*(6), 715–721. https://doi.org/10.1097/MCO.0b013e32833eebe5

Mesías, M., & Delgado-Andrade, C. (2017). Melanoidins as a potential functional food ingredient. *Current Opinion in Food Science*, *14*, 37–42. https://doi.org/10.1016/j.cofs.2017.01.007

Meyer, K. A., Kushi, L. H., Jacobs, D. R., Slavin, J., Sellers, T. A., & Folsom, A. R. (2000). Carbohydrates, dietary fiber, and incident type 2 diabetes in older women. *The American Journal of Clinical Nutrition*, *71*(4), 921–930. https://doi.org/10.1093/ajcn/71.4.921

Michalska, A., Honke, J., Łysiak, G., & Andlauer, W. (2016). Effect of drying parameters on the formation of early and intermediate stage products of the Maillard reaction in different plum (Prunus domestica L.) cultivars. *LWT - Food Science and Technology*, *65*, 932–938. https://doi.org/10.1016/j.lwt.2015.09.015

Miljkovic, D. (2011). Coffee cherry cosmetic composition and methods. *Dusan Miljkovic*.

Moreira, A. S. P., Nunes, F. M., Domingues, M. R., & Coimbra, M. A. (2012). Coffee melanoidins: structures, mechanisms of formation and potential health impacts. *Food & Function*, *3*(9), 903. https://doi.org/10.1039/c2fo30048f

Moreira, A. S. P., Nunes, F. M., Simões, C., Maciel, E., Domingues, P., Domingues, M. R. M., & Coimbra, M. A. (2017). Transglycosylation reactions, a main mechanism of phenolics incorporation in coffee melanoidins: Inhibition by Maillard reaction. *Food Chemistry*, *227*, 422–431. https://doi.org/10.1016/j.foodchem.2017.01.107

Murthy, P. S., & Madhava Naidu, M. (2012). Sustainable management of coffee industry by-products and value addition—A review. *Resources, Conservation and Recycling*, *66*, 45–58. https://doi.org/10.1016/j.resconrec.2012.06.005

Murthy, P. S., Manjunatha, M. R., Sulochannama, G., Madhava Naidu, M., Pushpa S. Murthy, M.R. Manjunatha, G. S. and M. M. N., Murthy, P. S., … Naidu, M. M. (2012). Extraction, Characterization and Bioactivity of Coffee Anthocyanins. *European Journal of Biological*

Sciences, *4*(1), 13–19. https://doi.org/10.5829/idosi.ejbs.2012.4.1.6149

Murthy, P. S., & Naidu, M. M. (2012). Recovery of Phenolic Antioxidants and Functional Compounds from Coffee Industry By-Products. *Food and Bioprocess Technology*, *5*(3), 897–903. https://doi.org/10.1007/s11947-010-0363-z

Mussatto, S. I., Carneiro, L. M., Silva, J. P. A., Roberto, I. C., & Teixeira, J. A. (2011). A study on chemical constituents and sugars extraction from spent coffee grounds. *Carbohydrate Polymers*, *83*(2), 368–374. https://doi.org/10.1016/j.carbpol.2010.07.063

Mussatto, S. I., Machado, E. M. S., Martins, S., & Teixeira, J. a. (2011). Production, Composition, and Application of Coffee and Its Industrial Residues. *Food and Bioprocess Technology*, *4*(5), 661–672. https://doi.org/10.1007/s11947-011-0565-z

Napolitano, A., Fogliano, V., Tafuri, A., & Ritieni, A. (2007). Natural occurrence of ochratoxin A and antioxidant activities of green and roasted coffees and corresponding byproducts. *Journal of Agricultural and Food Chemistry*, *55*(25), 10499–10504. https://doi.org/10.1021/jf071959+

Narita, Y., & Inouye, K. (2014). Review on utilization and composition of coffee silverskin. *Food Research International*, *61*, 16–22. https://doi.org/10.1016/j.foodres.2014.01.023

Natucafé S.A.S. (2019). Coffee Mucilage Concentrate. Retrieved from http://natucafe.co/coffee-mucilage-concentrate.php

Oliveira, L. S., & Franca, A. S. (2014). *An Overview of the Potential Uses for Coffee Husks*. *Coffee in Health and Disease Prevention*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-409517-5.00031-0

Orrego, D., Zapata-Zapata, A. D., & Kim, D. (2018). Optimization and scale-up of coffee mucilage fermentation for ethanol production. *Energies*, *11*(4), 1–12. https://doi.org/10.3390/en11040786

Pandey, A., Soccol, C. R., Nigam, P., Brand, D., Mohan, R., & Roussos, S. (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochemical Engineering Journal*, *6*(2), 153–162. https://doi.org/10.1016/S1369-703X(00)00084-X

Pearson, K. (2015). Nutraceuticals and skin health: what are the key benefits and protective properties? *Journal of Aesthetic Nursing*, *4*(4), 166–171. https://doi.org/10.12968/joan.2015.4.4.166

Pectcof B.V. (2019). Retrieved from https://pectcof.com/

Pedreschi, F., Mariotti, M. S., & Granby, K. (2014). Current issues in dietary acrylamide: Formation, mitigation and risk assessment. *Journal of the Science of Food and Agriculture*, *94*(1), 9–20. https://doi.org/10.1002/jsfa.6349

Porres, C., Alvarez, D., & Calzada, J. (1993). Caffeine reduction in coffee pulp through silage. *Biotechnology Advances*, *11*(3), 519–523. https://doi.org/10.1016/0734-9750(93)90020-N

Pourfarzad, A., Mahdavian-Mehr, H., & Sedaghat, N. (2013). Coffee silverskin as a source of dietary fiber in bread-making: Optimization of chemical treatment using response surface methodology. *LWT - Food Science and Technology*, *50*(2), 599–606.

https://doi.org/10.1016/j.lwt.2012.08.001

Prata, E. R. B. A. B. a, & Oliveira, L. S. (2007). Fresh coffee husks as potential sources of anthocyanins. *LWT - Food Science and Technology*, *40*(9), 1555–1560. https://doi.org/10.1016/j.lwt.2006.10.003

Ramalakshmi, K., Rao, L. J. M., Takano-Ishikawa, Y., & Goto, M. (2009). Bioactivities of low-grade green coffee and spent coffee in different in vitro model systems. *Food Chemistry*, *115*(1), 79–85. https://doi.org/10.1016/j.foodchem.2008.11.063

Ramirez-Coronel, M. A., Marnet, N., Kolli, V. S. K., Roussos, S., Guyot, S., & Augur, C. (2004). Characterization and estimation of proanthocyanidins and other phenolics in coffee pulp (Coffea arabica) by thiolysis-high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, *52*(5), 1344–1349. https://doi.org/10.1021/jf035208t

Ramirez-Martinez, J. R. (1988). Phenolic compounds in coffee pulp: Quantitative determination by HPLC. *Journal of the Science of Food and Agriculture*, *43*(2), 135–144. https://doi.org/10.1002/jsfa.2740430204

Ramirez Velez, A., & Jaramillo Lopez, J. C. (2015). Process for obtaining honey and/or flour of coffee from the pulp or husk and the mucilage of the coffee bean. US20150017270 A1. Colombia.

Rathinavelu, R., & Graziosi, G. (2005). Potential alternative use of coffee wastes and by-products, (942), 1–4.

Rebollo-Hernanz, M., Zhang, Q., Aguilera, Y., Martín-Cabrejas, M. A., & Gonzalez de Mejia, E. (2019). Phenolic compounds from coffee by-products modulate adipogenesis-related inflammation, mitochondrial dysfunction, and insulin resistance in adipocytes, via insulin/PI3K/AKT signaling pathways. *Food and Chemical Toxicology*, *132*, 110672. https://doi.org/10.1016/j.fct.2019.110672

Rios, M. B. (2018). *Application of coffee cascara as a source of dietary fiber in the preparation of gluten-free breads*. Universidad Politécnica de Madrid.

Rocha, M. V. P., de Matos, L. J. B. L., Lima, L. P. De, Figueiredo, P. M. D. S., Lucena, I. L., Fernandes, F. A. N., & Gonçalves, L. R. B. (2014). Ultrasound-assisted production of biodiesel and ethanol from spent coffee grounds. *Bioresource Technology*, *167*, 343–348. https://doi.org/10.1016/j.biortech.2014.06.032

Rodrigues, F., Pereira, C., Pimentel, F. B., Alves, R. C., Ferreira, M., Sarmento, B., … Oliveira, M. B. P. P. (2015). Are coffee silverskin extracts safe for topical use? An in vitro and in vivo approach. *Industrial Crops and Products*, *63*, 167–174. https://doi.org/10.1016/j.indcrop.2014.10.014

Rodrigues, F, Sarmento, B., Amaral, M. H., & Oliveira, M. B. P. P. (2016). Exploring the antioxidant potentiality of two food by-products into a topical cream: stability, in vitro and in vivo evaluation. *Drug Development and Industrial Pharmacy*, *42*(6), 880–889. https://doi.org/10.3109/03639045.2015.1088865

Rodrigues, Francisca, Matias, R., Ferreira, M., Amaral, M. H., & Oliveira, M. B. P. P. (2016). In vitro and in vivo comparative study of cosmetic ingredients Coffee silverskin and

hyaluronic acid. *Experimental Dermatology*, *25*(7), 572–574. https://doi.org/10.1111/exd.13010

Ronga, D., Pane, C., Zaccardelli, M., & Pecchioni, N. (2016). Use of Spent Coffee Ground Compost in Peat-Based Growing Media for the Production of Basil and Tomato Potting Plants. *Communications in Soil Science and Plant Analysis*, *47*(3), 356–368. https://doi.org/10.1080/00103624.2015.1122803

Sahasrabudhe, A., & Deodhar, M. (2010). Anti-hyaluronidase, anti-elastase activity of Garcinia indica. *International Journal of Botany*. https://doi.org/10.3923/ijb.2010.299.303

Sampaio, A., Dragone, G., Vilanova, M., Oliveira, J. M., Teixeira, J. a., & Mussatto, S. I. (2013). Production, chemical characterization, and sensory profile of a novel spirit elaborated from spent coffee ground. *LWT - Food Science and Technology*, *54*(2), 557–563. https://doi.org/10.1016/j.lwt.2013.05.042

Secret Cherry Tea. (2019). Superfood Coffee Cherry Tea. Retrieved from https://www.secretcherrytea.com/

Seo, J., Jung, J. K., & Seo, S. (2015). Evaluation of nutritional and economic feed values of spent coffee grounds and *Artemisia princeps* residues as a ruminant feed using *in vitro* ruminal fermentation. *PeerJ*, *3*, e1343. https://doi.org/10.7717/peerj.1343

Sleeth, M. L., Thompson, E. L., Ford, H. E., Zac-Varghese, S. E. K., & Frost, G. (2010). Free fatty acid receptor 2 and nutrient sensing: A proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation. *Nutrition Research Reviews*, *23*(1), 135–145. https://doi.org/10.1017/S0954422410000089

St-Pierre, D. H., Rabasa-Lhoret, R., Lavoie, M. È., Karelis, A. D., Strychar, I., Doucet, E., & Coderre, L. (2009). Fiber intake predicts ghrelin levels in overweight and obese postmenopausal women. *European Journal of Endocrinology*, *161*(1), 65–72. https://doi.org/10.1530/EJE-09-0018

Tarini, J., & Wolever, T. M. S. (2010). The fermentable fibre inulin increases postprandial serum short-chain fatty acids and reduces free-fatty acids and ghrelin in healthy subjects. *Applied Physiology, Nutrition, and Metabolism*, *35*(1), 9–16. https://doi.org/10.1139/H09-119

The Coffee Cherry Co. (2014). Retrieved from https://coffeecherryco.com/

The European Commission. (2012). Commission Regulation (EU) No 432/2012. *Official Journal of the European Union*, *13*(1924). Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32012R0432&from=EN

The European Parliament and The Council of the European Union. (2006). REGULATION (EC) No 1924/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on nutrition and health claims made on foods. *Official Journal of the European Union*, (404), 9–25. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1924&from=en

The European Parliament and The Council of the European Union. (2015). Regulation (Eu) 2015/2283 on novel foods. *Official Journal of the European Union*, *327*(258), 1–22.

Torres-León, C., Ramírez-Guzman, N., Londoño-Hernandez, L., Martinez-Medina, G. A., Díaz-

Herrera, R., Navarro-Macias, V., … Aguilar, C. N. (2018). Food Waste and Byproducts: An Opportunity to Minimize Malnutrition and Hunger in Developing Countries. *Frontiers in Sustainable Food Systems*, *2*(September), 1–17. https://doi.org/10.3389/fsufs.2018.00052

Toschi, T. G., Cardenia, V., Bonaga, G., Mandrioli, M., & Rodriguez-estrada, M. T. (2014). Coffee Silverskin : Characterization , Possible Uses , and Safety Aspects. *Journal of Agricultural and Food Chemistry*, *62*, 10836–10844.

US Department of Agriculture. (2015). *Scientific Report of the 2015 Dietary Guidelines Advisory Committee*. Retrieved from https://health.gov/dietaryguidelines/2015-scientific-report/pdfs/scientific-report-of-the-2015-dietary-guidelines-advisory-committee.pdf

USDA Foreign Agricultural Service. (2019). Coffee: World Markets and Trade. *Coffee: World Markets and Trade*. Retrieved from http://apps.fas.usda.gov/psdonline/circulars/coffee.pdf

Vazquez Sanchez, K., Martinez-Saez, N., del Castillo, M. D., & Campos-Vega, R. (2015). Antioxidant coffee dietary fiber for gastrointestinal health and diabetes. In D. M. Martirosyan (Ed.), *Functional and medical foods for chronic diseases: bioactive compounds and biomarkers*. The 20th International Conference, Boston, USA. 22-23 September, 2016.

Vilela, W. F., Leão, D. P., Franca, A. S., & Oliveira, L. S. (2016). Effect of Peroxide Treatment on Functional and Technological Properties of Fiber-Rich Powders Based on Spent Coffee Grounds. *International Journal of Food Engineering*, *2*(1). https://doi.org/10.18178/ijfe.2.1.42-47

WHO. (2003). Diet, nutrition and the prevention of chronic diseases. *World Health Organization Technical Report Series*, *916*, i-viii-1-149-backcover. https://doi.org/ISBN 92 4 120916 X ISSN 0512-3054 (NLM classification: QU 145)

Williamson, K., & Hatzakis, E. (2019). NMR analysis of roasted coffee lipids and development of a spent ground coffee application for the production of bioplastic precursors. *Food Research International*, *119*, 683–692. https://doi.org/10.1016/J.FOODRES.2018.10.046

World Health Organization (WHO). (2015). European Food and Nutrition Action Plan 2015 - 2020.

Yang, J., Ching, Y. C., & Chuah, C. H. (2019). Applications of lignocellulosic fibers and lignin in bioplastics: A review. *Polymers*, *11*(5), 1–26. https://doi.org/10.3390/polym11050751

Zhang, X., Tao, N., Wang, X., Chen, F., & Wang, M. (2015). The colorants, antioxidants, and toxicants from nonenzymatic browning reactions and the impacts of dietary polyphenols on their thermal formation. *Food and Function*, *6*(2), 345–355. https://doi.org/10.1039/c4fo00996g

HYPOTHESIS, OBJECTIVES AND WORK PLAN

1. Hypothesis

Coffee by-products represent an environmental problem and an inefficiently use of the natural resources worldwide. Their conversion into novel food ingredients avoiding the generation of further waste represents a great opportunity for the sustainability and competitiveness of the coffee sector; as well as, for achieving a sustainable nutrition and health.

2. Objectives

The **general objective** of this thesis was to validate the feasibility of coffee by-products (husk, parchment, silverskin and spent coffee grounds) as novel food ingredients for a sustainable nutrition and health following the Regulation (EU) 2015/2283.

In order to achieve this goal, the following **specific objectives** were proposed:

1. **To study the nutritional composition and safety of coffee cherry by-products generated in producing countries** *(Chapter 1, Study 1).*
   Two different ingredients were obtained from each coffee by-product by aqueous extraction, which is a sustainable and easy to scale-up process for generating ingredients avoiding new wastes. Chemical composition, antioxidant properties (*in vitro* and in cellular models) and safety (chemical, biological and processing contaminants; and acute toxicity assay according to the OECD Test Guidelines 425) of the proposed novel ingredients were assessed.
2. **To validate the novel ingredient obtained from the coffee roasting by-product (coffee silverskin extract, CSE)** *(Chapter 2).*
   This part includes the assessment of the genotoxicity and cytotoxicity of CSE, using the comet and the MTT assay, respectively *(Study 2).*In addition, the preventive potential against induced oxidative DNA damage was also studied. The nutritional value of the potential novel ingredient, CSE, its safety and effects of key biological functions were assessed *in vivo (Study 3).* To achieve this goal, a repeated dose study was carried out in Wistar rats and hormone secretion, antioxidant and anti-inflammatory biomarkers and fiber fermentation of the novel ingredient were analyzed in biological samples.
3. **To validate the coffee brewing by-product (spent coffee grounds, SCGs) as a safe, healthy and sustainable dietary fiber** *(Chapter 3, Study 4).*
   This study aimed to study the composition and *in vitro* bioaccessibility of lipids and diterpenes in SCGs, their impact on liver biomarkers and their excretion in

feces *in vivo.* The dietary fiber effect of SCGs was addressed by a radiographic study.

4. **To make a preliminary chemical characterization and to assess the health promoting properties of isolated biomolecules from coffee beans by-products (melanoidins)** *(Chapter 4).*
   To achieve this goal, the high molecular weight fraction was isolated from CSE and SCGs by ultrafiltration. Chemical characterization and antioxidant properties on healthy human colon cells were evaluated *(Study 5).* In addition, the structural and functional properties and the bioactivity of melanoidins from CSE was further studied *in vivo (Study 6).*

Our research group is an international reference in the study of the conversion of coffee by-products in novel ingredients (see "list of publications"). The most studied by-product has been coffee silverskin. Therefore, a review regarding the health promoting properties of this by-product was published (*Annex 1).* The main contributions presented in this thesis refer to the validation on coffee by-products as novel food ingredients. The application of CSE as a novel ingredient will be recently submitted (*Annex 2)*. However, the use of CSE as a dermaceutical ingredient for promoting skin health has been also studied (*Annex 3)* and approved by the International Cosmetic Ingredient Nomenclature Committee (*Annex 4*). Dermaceutical product development is being carried out in collaboration with industrial partners.

The work plan followed to achieve the main and specific objectives defined in this thesis is presented in Figure 1.

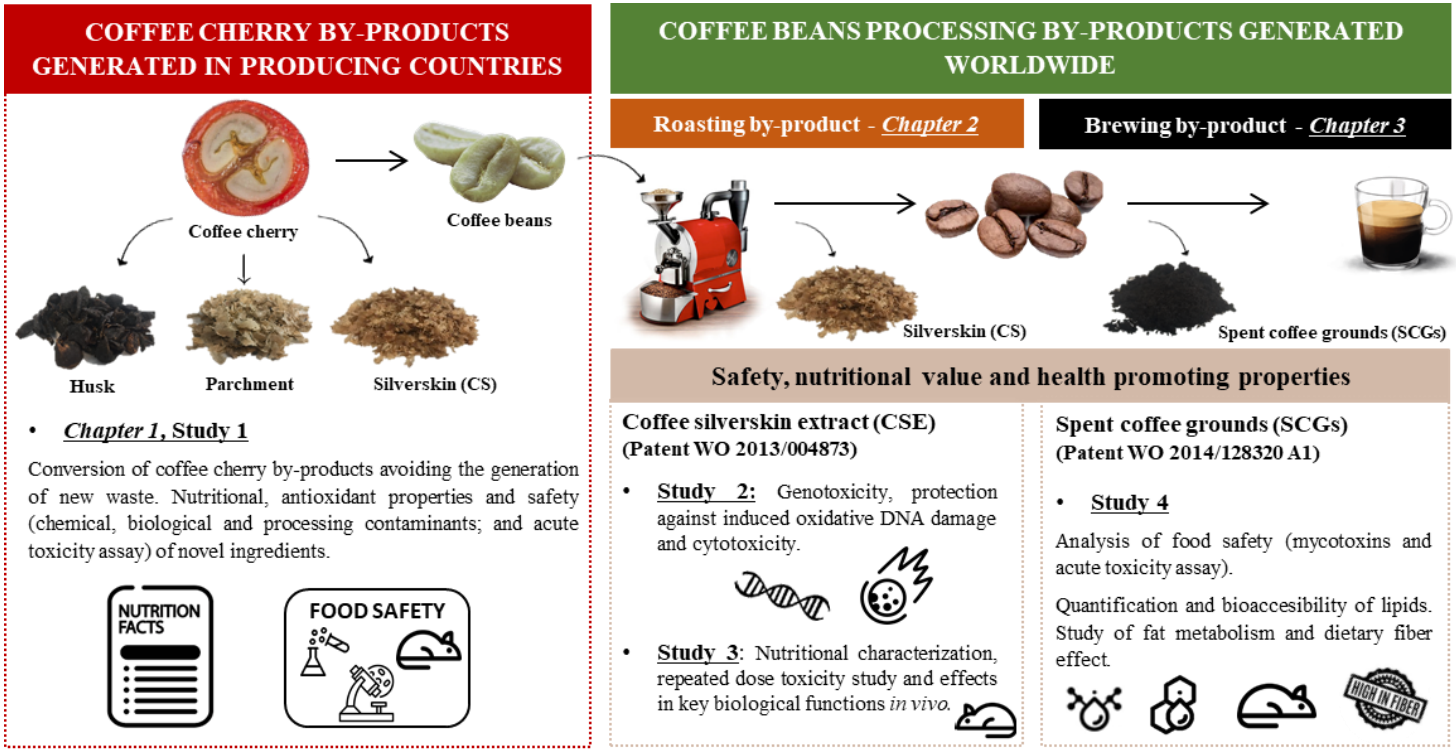


Figure 1. Schematic view of the workplan of the present study.

MAIN CONTRIBUTIONS

CHAPTER 1

Study 1: Validation of coffee by-products as novel food ingredients

This chapter provides a data on the chemical characterization, biological properties and safety of coffee by-products obtained in producing countries for their validation as food ingredients. Results from this chapter have been published in:

Iriondo-DeHond, A., Aparicio García, N., Velazquez Escobar, F., San Andres, M. I., Sanchez-Fortun, S., Blanch, G. P., Fernandez-Gomez, B., Guisantes Batan, E. & del Castillo, M. D. 2019. Validation of coffee by-products as novel food ingredients. *Innovative Food Science and Emerging Technologies*, 51, 194–204. Available at: https://doi.org/10.1016/j.ifset.2018.06.010

Validation of coffee by-products as novel food ingredients

Amaia Iriondo-DeHond [1], Natalia Aparicio García [1], Beatriz Fernandez-Gomez [1], Eduardo Guisantes-Batan [2], Francisco Velázquez Escobar [3], Gracia Patricia Blanch [4], Manuel Ignacio San Andres [5], Sebastián Sanchez-Fortun [5], María Dolores del Castillo [1]

[1] Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM), C/ Nicolás Cabrera, 9, Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain.

[2] Universidad de Castilla-La Mancha, Instituto Regional de Investigación Científica Aplicada (IRICA), Av. Camilo José Cela s/n, 13071 Ciudad Real, Spain.

[3] Technische Universität Berlin, 135/PC14 Max Volmer Laboratorium für biophysikalische Chemie, 1023 Berlin, Germany.

[4] Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN) (CSIC), C/ José Antonio Novais, 10, 28040 Madrid, Spain.

[5] Facultad de Veterinaria, Universidad Complutense de Madrid, Av. Puerta de Hierro, s/n, 28040 Madrid, Spain.

Innovative Food Science and Emerging Technologies, 2019, 51, 194–204.
https://doi.org/10.1016/j.ifset.2018.06.010

Abstract

This research aimed to validate coffee husk, parchment and silverskin as new health-promoting food ingredients. Characterization of the novel ingredients was carried out by Raman and infrared spectroscopy and analysis of total phenolic compounds, chlorogenic acid, caffeine and dietary fiber. Antioxidant properties of the novel ingredients were tested by ABTS and intracellular ROS formation in HepG2 cells. Pesticides, mycotoxins, acrylamide and acute toxicity experiments following OECD Test Guidelines 425 were performed to assess the food safety of extracts, solid residues and raw materials. Husk and silverskin are proposed as a source of two food ingredients: aqueous extracts enriched in phytochemicals and antioxidant dietary fiber while parchment is proposed as a natural source of antioxidant dietary fiber. No lesions were found in selected isolated vital organs from treated animals. Coffee by-products can be converted into safe food ingredients allowing a whole food waste recovery. Analyses of contaminants are essential for achieving this goal.

Keywords: antioxidants; coffee by-products; dietary fiber; food safety; novel food ingredient; sustainability

1. Introduction

Coffee is a cherry formed by various anatomic parts with distinct morphologies (Figure 1) and unique chemical compositions. It comprises a plethora of nutrients and bioactive phytochemicals with diverse preventive and therapeutic effects for chronic diseases (del Castillo *et al*., 2017). Nowadays, coffee beans are almost exclusively used for the preparation of the beverage. While the coffee industry is very diverse, coffee processing can generally be described by ten steps: planting, harvesting and processing the cherries, drying, milling and trading, tasting, roasting, grinding and finally brewing. Even though most of the health-promoting phytochemicals and nutrients are found in the coffee cherry, 90 % of the cherry is discarded during processing as agricultural waste or by-product (del Castillo *et al*., 2018).

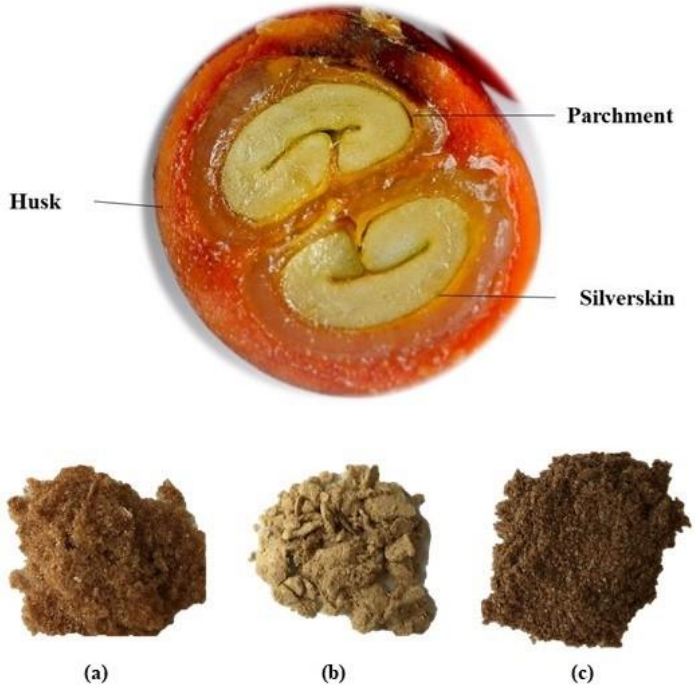


Figure 1. Transversal section of a ripe coffee cherry, showing its anatomic parts; and powdered aqueous extracts obtained from Arabica (a) husk, (b) parchment and (c) CS following the procedure described in the patent WO 2013/004873.

According to the European Commission, a "food waste" is defined as "food (including inedible parts) lost from the food supply chain, not including food diverted to material uses such as bio-based products, animal feed, or sent for redistribution" (European Commission, 2014). The coffee industry is responsible for the generation of large amounts of residues that represent a great pollution hazard if discharged into the environment (Chanakya & De Alwis, 2004). The future of coffee depends on sustainable practices, and a wide range of initiatives can be implemented in every part of the coffee supply chain: improving the conditions at origin, recycling packaging materials, reducing emissions, developing eco-friendly facilities or designing new coffee products. The conversion of coffee waste into food ingredients is a research priority in the Nutrition and Food Chemistry field.

Esquivel and Jiménez (2012) defined coffee husk as the outer skin and pulp obtained from the wet processing of coffee berries (Esquivel & Jiménez, 2012). Coffee husk encloses the coffee beans and compromises nearly 45 % of the berry. It has a high content in carbohydrates (35 – 85 %), soluble fibers (30.8 %), minerals (3 – 11 %) and proteins (5 – 11 %). It is also rich in insoluble dietary fiber and can be a source of phytochemicals such as tannins (5 – 9 %) and cyanidins (20 %) for the food and pharmaceutical industries (del Castillo *et al*., 2018; Esquivel & Jiménez, 2012). Phytochemicals have recently received considerable interest because of their safety and potential positive physiological effects on the human body (Singh & Geetanjali, 2013). To date, coffee husk has been used as a source of dietary fiber (www.pectcof.com) and also as a source of anthocyanins (Murthy *et al*., 2012). Considering its chemical composition and the proposed applications, coffee husk has a great potential as a food ingredient and as a natural source of nutrients and bioactive compounds.

Parchment is a strong fibrous endocarp that covers both hemispheres of the coffee seed and separates them from each other (Belitz *et al*., 2009). It represents 5.8 % of berry dry weight and is formed by (α-) cellulose (40 – 49 %), hemicellulose (25 – 32 %), lignin (33 – 35 %) and ash (0.5 – 1 %) (del Castillo *et al*., 2018). In wet coffee processing, the parchment is removed after drying and hulling, which allows it to be collected and used separately from other by-products (Esquivel & Jiménez, 2012). To date, no applications have been found for coffee parchment as a food ingredient.

Coffee silverskin (CS) represents 4.2 % (w/w) of the coffee cherry and constitutes a thin tegument of the outer layer of the two beans forming the green coffee seed. It is obtained as a by-product of the roasting process. CS has a high dietary fiber content (68 – 80 %), polysaccharides are also abundant components (60 – 70 %) and total sugar content varies

greatly (1.6 – 12 %) (Pourfarzad *et al*., 2013). CS contains protein, fat, and ash, at 16.2 – 19.0 %, 1.56 – 3.28 %, and 5 – 7 %, respectively (del Castillo *et al*., 2018). Borrelli and colleagues found that CS, obtained from several Italian roasting plants, had a high amount of soluble dietary fiber (about 14 % of the total fiber) and a very high antioxidant activity (Borrelli *et al*., 2002). Previous studies have suggested the use of CS as a source of dietary fiber and prebiotics (Ballesteros *et al*., 2014; Borrelli *et al*., 2004).

In 2016, the European Food Safety Authority (EFSA) reported that only 1.5 % of 83,000 food samples analyzed exceeded the legal limit of pesticides, and the national competent authorities had to take appropriate enforcement actions (European Food Safety Authority (EFSA), 2016). To date, no studies have analyzed pesticide content in coffee byproducts. Moreover, coffee is susceptible to contamination by mycotoxins, toxic compounds that result from fungal secondary metabolism under certain conditions, which cause different toxicological effects in humans (Afsah-Hejri *et al*., 2013; Gamboa-Gaitán, 2012). The most studied mycotoxin in coffee is ochratoxin A (OTA), the only mycotoxin subjected to European legislation (European Commission (EC) 1881/2006). Other contaminants may also be formed during food processing, *e.g.* Maillard reaction products such as acrylamide. This low molecular weight compound is highly soluble in water, and has a well-documented carcinogenic potential (European Commission, 2017). It is formed in food processing as a degradation product of asparagine and sugars, typically at 120 °C and low moisture (European Commission, 2017). Therefore, it could be found in roasted coffee beans and CS. Previous studies have confirmed the presence of acrylamide in CS (333 μg/kg) (Garcia-Serna *et al*., 2014) in the same amount as that reported in roasted coffee and approximately 10 times lower than that reported in instant coffee (European Commission, 2017).

The aim of this research was to perform a comparative study of the three coffee by-products obtained before (husk and parchment) and after the roasting process (silverskin); validate their potential and safety as sustainable food ingredients; explore novel applications for them through environment-friendly procedures and implement novel analytical tools such as vibrational spectroscopy.

2. Materials and methods

2.1. Reagents

Chlorogenic acid, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), caffeine, Folin-Ciocalteu reagent, tert-butyl hydroperoxide (tBOOH), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazole-y)-2,5-

diphenyltetrazolium bromide (MTT) and 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical (Sigma-Aldrich, St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Lonza (Basel, Switzerland).

2.2. Raw materials

Coffee husk and parchment from Arabica species were kindly provided by Delikia from Finca Morenitas in Nicaragua. CS from Arabica and Robusta species from Colombia was provided by Fortaleza S.A. (Spain). All coffee by-products were derived from wet-processing.

2.3. Raman and infrared spectroscopy

Fourier-Transform (FT) Raman spectra were recorded with an RFS-100/S spectrometer (Bruker, Billerica, USA). A single 4x4 mm^2 endocarp shell was positioned between a thin quartz window (1 mm) and a silver-mirror. Spectra were recorded according to previous measuring protocols for green coffee (Keidel *et al*., 2010).

Complementary endocarp FT-infrared (IR) spectra were recorded in a Tensor27 FT-spectrometer (Bruker, Billerica, USA), equipped with a diamond attenuated total reflection (ATR) cell (Durascope Resultec). The single endocarp shell was positioned and pressed towards the ATR-diamond surface. Absorbance spectrum was calculated from the recorded sample spectrum and a previously measured reference spectrum (empty ATR-cell). Further details regarding spectrometers and data processing are given elsewhere (Ulijasz *et al*., 2009).

For the band assignment of the endocarp IR and the Raman spectra, we refer to previous experimental works in cellulose, lignin and further related compounds (Adebajo *et al*., 2006; Gierlinger *et al*., 2013; Nabais *et al*., 2008).

2.4. Characterization of coffee by-product extracts

2.4.1. Preparation of aqueous extracts from coffee by-products

Coffee by-product extracts were produced as described in the patent WO 2013/004873 (del Castillo *et al*., 2013). Briefly, 50 mg of coffee husk, parchment or silverskin were added per milliliter of boiling water. This mixture was stirred at 250 rpm for 10 minutes; filtered by a 250 µm filter and then through Whatman paper No. 4; and the filtrate and the insoluble residue were freeze-dried. Extracts were prepared three times.

2.4.2. Caffeine and 5-CQA

Caffeine and 5-CQA content was determined by ultra-performance liquid chromatography - tandem mass spectrometer (UPLC-MS/MS) (Thermo-Scientific, San José, CA, USA) as described by Fernandez-Gomez *et al*. (2016). For quantification, samples were diluted in milli-Q water and added with 50 µg/ml of phluoroglucinol as internal standard for the most abundant chlorogenic acid (CGA) described for coffee (5-caffeoylquinic acid, 5-CQA) and 50 µg/ml of salicylic acid as internal standard for caffeine. To verify the reproducibility and possible variations, all experiments were performed in triplicate. Compound concentrations were expressed in mg caffeine or 5-CQA/ g of sample.

2.4.3. Overall antioxidant capacity

The trapping capacity of cationic free radicals was evaluated using the method of radical $ABTS^{+}$ bleaching described by Re *et al*., (1999) and modified by Oki *et al*. (2006) for its use in microplate. Aqueous solutions of CGA (0.15 – 2.0 mmol/l) were used for calibration. Absorbance was measured in microplate using a UV-Visible Spectrophotometer (BioTek Instruments, Winooski, VT, USA). All measurements were performed in triplicate, and results were expressed as mg CGA eq. per gram of sample.

2.4.4. Total phenolic compounds

Folin–Ciocalteu adapted to a micromethod format was used for the analysis of total phenolic compounds in samples (Contini *et al*., 2008). The reaction was initiated by mixing 10 µl of sample with 150 µl of Folin–Ciocalteu solution. After 3 minutes at room temperature, 50 µl of sodium bicarbonate solution were added. The kinetics of the reaction at 37 ºC was followed for 120 minutes by measuring the absorbance at 735 nm once every minute using a UV-Visible Spectrophotometer (BioTek Instruments, Winooski, VT, USA). Sample blank and reagent blank were also analyzed in each set of samples. A CGA calibration curve was used for quantification (0.1 – 0.8 mg/ml). Results were expressed as µmol CGA eq./g. All measurements were performed in triplicate.

2.4.5. Total flavonoid content

Total flavonoid content was determined using a previously described colorimetric method (Xu & Chang, 2007). Briefly, 100 µL of samples or rutin (RUT) standard solution were mixed with 30 µL of a 5 % $NaNO_2$ solution. After 5 minutes of incubation, 30 µL of a 10% $AlCl_3$ solution were added and allowed to stand for another 6 minutes before adding 100 µL of 2M NaOH. Absorbance was measured immediately at 510 nm

using a UV-Visible Spectrophotometer (BioTek Instruments, Winooski, VT, USA). Results were calculated and expressed as milligrams of RUT equivalents per 100 mg of extract. The determination was carried out in triplicate.

2.4.6. Cell culture and treatments

HepG2 human hepatocyte cell line was kindly provided by Dr. Paloma Morales (Facultad de Veterinaria, Universidad Complutense de Madrid, Spain). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine and 1 % penicillin/streptomycin in standard conditions (37 ºC, 5 % CO_2, in a humidified incubator, BINDER CB series 2010, Tuttlingen, Germany).

2.4.7. Cell viability assays

The effect of different concentrations of husk, parchment, and CS extracts on cell viability was measured using the MTT assay (Bakondi *et al*., 2003). Powdered extracts were prepared in PBS and sterile filtered when used in cell culture. Cells were cultured at a density of 1.0 x 10^5 cells per well of a 96-well plate. After 24 hours, cells were treated with husk, parchment, and CS extracts diluted in DMEM culture medium (0.1, 1, 10 and 20 mg/ml) for 24 hours. DMSO (50 %) was used as death control. Subsequently, cells were incubated in MTT solution (0.5 mg/ml) for 1 h at 37 ºC. The supernatant was then removed, 100 µL of DMSO were added, and the optical density at 570 nm was measured using a microplate reader (BioTek Synergy HT Multi-Mode Microplate Reader, Winooski, VT, USA). Experiments were carried out in triplicate.

2.4.8. Intracellular ROS scavenging assay

The analysis was performed by measuring the fluorescence intensity of the DCFH-DA probe, which was proportional to the amount of ROS formed (Gomes *et al*., 2005). A 10 mM solution of DCFH-DA was prepared (5 mg in 1 ml DMSO), and a 50 µL aliquot was separated. Then, 800 µL of DMSO were added to the 50 µL solution. After 24 hours of extract incubation, cells were pre-loaded with 2.5 µL/well of this last solution for 30 minutes at 37 ºC. After incubation, DCFH becomes dichlorofluorescein (DCF) due to intracellular oxidants and will emit fluorescence. Next, the culture medium was removed; cells were washed with PBS; and the three coffee by-product extracts (1 mg/ml) were added for 1 hour. tBOOH 1 mM was used as a positive control. Then, fluorescence was measured at 485 nm/528 nm (BioTek Synergy HT Multi-Mode Microplate Reader). Experiments were carried out in triplicate.

2.5. Characterization of the insoluble fraction obtained from coffee by-products during the extraction process

2.5.1. Dietary fiber

Insoluble (IDF), soluble (SDF) and total (TDF) dietary fiber content was determined using the Total Dietary Fiber Assay Kit (Megazyme International Ireland, Ireland) following the manufacturer's instructions, and based on the enzymatic–gravimetric method. Results are expressed as percentage (%).

2.5.2. Total protein

Protein content was determined by Kjeldahl mineralization followed by a colorimetric analysis of nitrogen for quantification (AOAC-32.1.22, 920.87). NH_4Cl was used for a standard calibration curve. A conversion factor (6.25) was used to calculate protein content. Results were expressed as % dry matter (d.m.).

2.5.3. Total lipid content

Using a modified protocol described by Folch *et al*. (1987), lipid content was determined by a solid-liquid extraction with n-hexane. Fifty milliliters of n-hexane were added to the insoluble fractions (1 g) and the mixture was homogenized using an Ultra-Turrax for 3 minutes and subjected to an ultrasonic bath for 20 minutes to improve lipid extraction. Then, samples were centrifuged at 1620 g for 20 minutes. Supernatant's solvent was then gently removed under controlled vacuum. Total lipid content was obtained by weighing difference. The assay was performed in duplicate and the results expressed as % lipid content.

2.5.4. Overall antioxidant capacity

Direct $ABTS^+$ assay was carried out according to Açar *et al*. (2009). Ten mg of sample were mixed with 90 mg of cellulose and stirred, 10 mg of the mixture were mixed together with 1.7 ml of $ABTS^+$ solution in a thermomixer (25 ºC, 2 minutes, 600 rpm). After centrifugation, absorbance of the supernatant was measured in a microplate. A CGA calibration curve (0 – 250 µg/ml) was used. Measurements were performed in triplicate and results expressed as mg CGA eq. per gram of sample.

2.6. Safety assays

2.6.1. Pesticides

The gas chromatography system (HPGC-7890A) was equipped with split/splitless injector and a MS detector (model MS-5975-VL-MSD with Triple –Axis Detector). The automatic split/splitless injector operated at 250 ºC and the split (1:10) was used to detect the analytes with helium as the carrier gas at an approximate flowrate of 1 ml/min. Gas chromatographic analysis of samples (husk extract and insoluble fraction, raw parchment and Arabica CS extract and insoluble fraction) was performed using a 30 m × 0.25 mm ID capillary fused silica column coated with a 0.25 µm film of TRB-5 (Teknokroma, Spain). Oven temperature-ramp followed a 12 °C/min increase-rate from 60 ºC (2 min) to 140 ºC and 6 °C/min up to 320 ºC. Desired temperature was maintained for 5 minutes.

The ion source temperature of the MS was 230 ºC, mass range 40–500 amu, scan speed 1666 amu/s, interface temperature 230 ºC. First, the SCAN mode and then the selected ion monitoring (SIM) mode were used for the analyses. An advanced pressure control supplied helium to the interface at constant pressure (95 kPa). In all cases, analyses were made in triplicate. Pesticide identification was based on the retention time and the relative abundance of the specific ions (used in the SIM mode), compared from the sample extract (Supplementary Material, Table S1). The number of monitored ions, preferably and if possible a minimum of three, was dependent on the pesticide. Analyzed pesticides were HCH-α, HCH-β, HCH-γ, HCH-δ, heptachlor, Aldrin, hexachlorbenzene, endosulfan-α, endosulfan-β, chlorpyrifos methyl, parathion methyl, parathion ethyl, dieldin, endrin, 4,4-DDE, 2,4-DDD, 44DDT, 24DDE and mirex.

2.6.2. Mycotoxins

Samples (husk extract and insoluble fraction, raw parchment and Arabica CS extract and insoluble fraction) were ground with an A-10 Basic laboratory mill (IKA, Staufen, Germany) and stored at -20 ºC until use. Ground samples (1 g) where mixed with acetonitrile:water (80:20, v/v, 2.5 ml) in a 15 ml centrifuge tube, vortexed (5 min), sonicated (3 min), vortexed again (1 min) and then samples were centrifuged (3000 g, 10 min). Subsequently, the supernatant was separated. The residue was extracted again following the same procedure and the supernatants were combined. One ml of the supernatant was transferred to a new 15 ml centrifuge tube and diluted with water (7 ml) to a total volume of 8 ml and centrifuged (3000 g, 10 min).

Mycotoxins were extracted using ISOLUTE® Myco 60 mg/3 ml (Biotage, Sweden) SPE columns following the procedure recommended by the supplier. Briefly, SPE column was conditioned with 2 ml acetonitrile, equilibrated with 2 ml of ammonium acetate (10 mM) and 3 ml of the diluted sample extract were loaded. Then, the column was washed with 10 mM ammonium acetate (3 ml) and with 10 mM ammonium acetate:acetonitrile (90:10, v/v, 3 ml) and dried for 30 seconds at maximum vacuum. Finally, the sample was eluted with 0.1% formic acid in acetonitrile (2 ml) and then with 0.1% formic acid in methanol (2 ml). The combined eluates were dried under vacuum (5 mbar at 35 °C) and reconstituted in 250 µL of 0.1 % acetic acid in 20 % acetonitrile:methanol (1:1, v/v).

HPLC-QToF analysis: The analytical system used consisted of a 1260 Infinity HPLC system coupled to a 6545 quadrupole-time of flight (Q-ToF) mass spectrometer detector (Agilent, Waldbronn, Germany). Control software was Mass Hunter Workstation (version B.06.11). The Q-ToF used a Dual Jet Stream Electrospray Ionization (Dual AJS-ESI) source operated in the positive ionization mode, and the following parameters were set: capillary voltage, 4000 V; fragmentor, 120 V; nozzle voltage 500 V; gas temperature, 130 ºC; drying gas,13 l/min; nebulizer, 30 psig; sheath gas temperature, 300 ºC; sheath gas flow, 11 l/min; acquisition range, 80-1000 m/z. Samples were analyzed after injection (30 µl) on a Zorbax Eclipse Plus C18 Rapid Resolution HD column (2.1 × 50 mm, 1.8 µm, Agilent, Santa Clara, CA) protected with a 5 mm guard column of the same material thermostated at 30 ºC. The solvents system were 5 mM ammonium formate + 0.1% formic acid (solvent A) and 5 mM ammonium formate + 0.1% formic acid in methanol (solvent B). The elution gradient was (time, % of solvent A): 0 min, 90 %; 0.5 min, 90 %; 10 min, 30 %; 15 min, 2 %; 18 min, 2 %; 20 min, 90 % and a post time of 5 min. Compounds were identified and quantified using the algorithm "Find by Formula" that evaluated the mass accuracy together with the isotopic relative abundance and isotopic separation.

2.6.3. Acrylamide

The quantification of acrylamide in Arabica CS extract and insoluble fraction was carried out in a laboratory that participates in appropriate proficiency testing schemes (which comply with the 'International Harmonised Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories') developed under the auspices of IUPAC/ISO/AOAC and using approved analytical methods for detection and quantification (HPLC-MS/MS, ISO 18862:2016) according to Commission Regulation (EU) 2017/2158. This analysis was performed by Coffee Consulting S.L.

2.6.4. Acute toxicity assay

In accordance to OECD (Organization for Economic Co-operation and Development) Test Guidelines 425 (Up and Down Procedure), healthy young adult nulliparous and nonpregnant female rats, weighing 180.52 ± 6.42 g (8 weeks old) at the start of the experiment, were procured from Charles River (Sant Cugat del Vallés, Spain). The present study was approved by the Institutional Animal Ethics Committee (Reg. No. PROEX 011/17) of Community of Madrid, Spain.

Rats were housed in cages with free access to standard food (A04 Safe Diets, Augy, France) and water *ad libidum*. According to OECD Test Guidelines 425 (OECD, 2008), limit test of raw parchment (section 2.2) and husk and Arabica CS extracts (section 2.4.1) was performed at 2000 mg/kg b.w. by gavage as a single dose to one rat. Husk and CS extracts were dissolved in water and parchment was dissolved in corn oil. As a control, one rat was dosed with water and another rat with corn oil to establish a comparative negative control group. Rats were closely observed for the first 30 minutes, then for 4 h. After survival of the treated rat, 4 additional female rats per group were administered with the same dose under the same conditions.

Body weight changes, signs of toxicity, behavior and mortality were observed for 24 hours after administration and once daily for 14 days. Then, rats were sacrificed by exposure to excess carbon dioxide in a gas chamber for necropsy examination. Internal organs (heart, lungs, liver, kidneys, spleen, adrenals, sex organs and brain) were collected for histopathological examination.

Tissue samples were routinely processed for histology and fixed in buffered formalin 10% (Panreac©, Barcelona, Spain, stabilized with methanol at pH 7) for 24 hours at room temperature. Samples were then embedded in synthetic paraffin (Casa Alvarez, Madrid, Spain) with a melting point of 56 °C, using an automatic tissue processor (ASP300, Leica®, Wetzlar, Germany), with a program of automatic transmissions alcohols increased histological grading. Blocks were performed in a block forming unit (console Leica© EG1140H and cold plate Leica© EG1130) and 4 micron thick sections were obtained from rotation microtome Leica© brand, model RM 2155. Sections were deparaffinized in xylene and hydrated in alcohol and water. Conventional staining method, hematoxylin & eosin, was used by means of Leica© auto stainer SP4040. Then, dehydrated first ascending alcohol series and xylene were used, and finally, mounted with DPX (Nustain©, Nottingham, UK).

2.7. Statistical analysis

Data were expressed as the mean ± SD. Prior to statistical analysis, all data were tested for variance homogeneity using the Levene's test. Logarithmic transformation of the response variable was used to achieve homocedasticity when needed. One-way analysis of variance (ANOVA) was performed. Statistical comparisons of the different treatments were performed using Tukey's test. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS Statistics 24.

3. Results and discussion

3.1. Characterization of coffee husk and its potential applications

Coffee husk extract (Figure 1a) showed the highest extraction yield (28 %), followed by CS extract (14 %) and parchment extract (2.3 %). Data on the characterization of coffee husk extract are summarized in Table 1. 5-CQA and caffeine were detected in all three extracts. Thus, caffeine concentration was higher than that corresponding to the phenolic compound whose concentration increased towards the fruit's core. Therefore, extract from coffee husk, the outer part of the cherry, showed the lowest values of the two bioactive compounds (1.7 mg 5-CQA/g and 13.9 mg caffeine /g). Husk extract also presented the lowest ($p < 0.05$) amount of total phenolic compounds (TPC) and total flavonoid content (TFC) under the tested conditions (15.6 mg CGA eq./g and 0.9 g RUT eq./g, respectively). The lowest total antioxidant capacity (TAC) corresponded to husk extract, in accordance with the obtained data of TPC, TFC and 5-CQA content. Some authors have previously reported the amount of caffeine and CGA in raw coffee husk, 13 and 25 mg/g, respectively (Murthy & Naidu, 2012; Pandey *et al*., 2000). Husk extract obtained under the conditions described in this work (WO 2013/004873) presented the same levels of caffeine as in the starting material.

Table 1. Bioactive compounds of Arabica husk, Arabica parchment and Arabica and Robusta silverskin (CS) extracts.

Analysis	Husk	Parchment	Silverskin	
			Arabica	Robusta
5-CQA (mg/g)	1.7 ± 0.3^{c}	6.1 ± 0.7^{b}	9.4 ± 2.6^{b}	21.3 ± 6.2^{a}
Caffeine (mg/g)	13.9 ± 0.8^{c}	58.2 ± 4.2^{a}	24 ± 1.3^{b}	53.3 ± 1.7^{a}
TPC (mg CGA eq./g)	15.6 ± 6^{c}	68.2 ± 12^{a}	44.8 ± 11.9^{b}	56.5 ± 10.7^{a}
TFC (g RUT eq./g)	0.9 ± 0.1^{c}	6 ± 0.1^{a}	3.4 ± 0.1^{b}	6.3 ± 0.3^{a}
TAC (mg CGA eq./g)	80.6 ± 9^{c}	202.2 ± 39.3^{a}	138.7 ± 14.8^{b}	169.5 ± 26.7^{b}

Data are expressed as the means ± standard deviation (n = 3). Values in each row with different letters differ significantly (Tukey test, $p < 0.05$). 5-CQA, 5-caffeoylquinic acid; CGA, chlorogenic acid; RUT, rutin; TAC, total antioxidant capacity; TFC, total flavonoid content; TPC, total phenolic compounds.

Table 2 shows the viability of HepG2 cells after 24 hours of incubation with husk extract at different concentrations (0.1 to 20 mg/ml). At concentrations of 0.1 and 1 mg/ml, cell viability was not significantly ($p > 0.05$) altered, while 20 mg/ml resulted cytotoxic. To date, there are no published studies about the effect of water soluble bioactive compounds from coffee husk on cell viability. Table 2 also shows the effect of husk extract on the prevention of intracellular ROS formation. The addition of tBOOH 1 mM to the culture medium significantly increased ($p < 0.05$) intracellular ROS production (≈ 44 %) compared to non-treated cells. Cells pre-treated for 24 hours with husk extract (1 mg/ml) showed a significant reduction ($p < 0.05$) in ROS levels compared to cells treated with tBOOH. ROS values were similar for non-treated cells (control) and those treated with the extract in absence of tBOOH, suggesting no effect in the physiological production of radicals. This is the first time that the effect of coffee husk on intracellular ROS formation has been described. Understanding the cellular defense action mechanism requires further research beyond the scope of the current study. Nevertheless, coffee husk extract should be considered a potential agent for the prevention of cellular damage induced by oxidative stress.

Table 2. Effect of 24 h treatment with noted concentrations of husk, parchment and CS extract on cell viability and intracellular ROS generation in HepG2 cells determined by the MTT assay and the DCFH-DA probe, respectively. DMSO (50 %) was used as a death control and oxidative damage was induced by t-BOOH (1 mM).

		% Cell Viability	**% ROS**
Control	-	100 ± 6.9^{d}	100 ± 14.3^{b}
Husk extract	0.1 mg/ml	96.5 ± 7.1^{d}	N.D.
	1 mg/ml	94.8 ± 9.2^{d}	84.9 ± 9.1^{b}
	10 mg/ml	92.4 ± 8.2^{cd}	N.D.
	20 mg/ml	80.8 ± 9.5^{c}	N.D.
Parchment extract	0.1 mg/ml	93.4 ± 20.7^{cd}	N.D.
	1 mg/ml	96.5 ± 22.9^{d}	54.6 ± 12.5^{a}
	10 mg/ml	21.1 ± 3.9^{b}	N.D.
	20 mg/ml	21.2 ± 6.3^{b}	N.D.
CS extract	0.1 mg/ml	96.5 ± 4.8^{d}	N.D.
	1 mg/ml	101.5 ± 3.9^{d}	63.9 ± 7.2^{a}
	10 mg/ml	115.8 ± 5.1^{e}	N.D.
	20 mg/ml	115.7 ± 11.5^{e}	N.D.
Death control	DMSO 50 %	4.8 ± 0.6^{a}	-
Oxidation control	tBOOH 1 mM	-	144.5 ± 24.2^{c}

N.D. non determined. Data represent means ± SD of 18 samples per condition. Different letters denote statistically significant differences between all treatments (Tuckey test, $p < 0.05$).

Soluble dietary fiber (SDF) was found in the insoluble fraction generated during the extraction process of coffee husk in significantly higher concentrations ($p < 0.05$) than in the rest of the studied samples (12.3 %, Table 3). Total dietary fiber (TDF) values of husk insoluble residue are in the range described by Gouvea *et al*. (2009), but in lower amounts compared to the results obtained by Navya & Pushpa (2013). Coffee husk has been characterized as a source of soluble dietary fiber aimed for use in food and pharmaceutical applications. Due to its properties as an emulsifier and stabilizer, this extracted dietary fiber is a promising new ingredient for the food and beverage industry (www.pectcof.com).

Table 3. Characterization of insoluble fractions recovered from Arabica husk, Arabica parchment and Arabica and Robusta silverskin (CS) during the aqueous extraction process.

Analysis	Husk	Parchment	Silverskin	
			Arabica	Robusta
TDF (%)	71.9 ± 5^{b}	92.6 ± 0.4^{a}	67.7 ± 1.6^{b}	69.3 ± 0.7^{b}
SDF (%)	12.3 ± 2.8^{a}	0.5 ± 0.1^{c}	3.7 ± 0.0^{b}	3.4 ± 0.6^{b}
IDF (%)	59.6 ± 2.2^{b}	92.1 ± 0.3^{a}	64 ± 1.6^{b}	66 ± 0.1^{b}
Total proteins (%)	8.4 ± 0.7^{b}	3.1 ± 0.2^{c}	10.9 ± 0.9^{b}	19 ± 1.8^{a}
Lipids (%)	2.7 ± 0.1^{b}	0.3 ± 0.0^{c}	2.6 ± 0.3^{b}	3.6 ± 0.3^{a}
TAC (mg CGA/g)	48.6 ± 2.5^{a}	4.1 ± 0.1^{d}	34.1 ± 2.5^{b}	22.4 ± 3.7^{c}

Data are expressed as the means ± standard deviation (n = 3). Values in each row with different letters differ significantly (Tukey test, $p < 0.05$). CGA, chlorogenic acid; IDF, insoluble dietary fiber; SDF, soluble dietary fiber; TAC, total antioxidant capacity; TDF, total dietary fiber**.**

In addition, husk residue presented a protein content of 8.4 % (Table 3), which is within the range reported by other authors (Brand *et al*., 2001; Gouvea *et al*., 2009). Lipid content was also in the same range reported by Gouvea *et al*. (2009). Regarding the total antioxidant capacity of this (water-insoluble) fraction, coffee husk showed the highest values, with 48.6 mg CGA/g ($p < 0.05$). This is the first time the antioxidant character of the dietary fiber from coffee husk is described. In addition to its antioxidant properties, consumption of SDF has nutritional relevance due to its positive impact on health by decreasing serum cholesterol, postprandial blood glucose and insulin contents in the human body (Bruntha Devi *et al*., 2011). The ability of SDF to retard the absorption of glucose in the small intestine is a desirable characteristic in the development of foods for diabetic populations (Onyango *et al*., 2004).

With regard to contaminants, no pesticides were detected either in husk extract or husk insoluble fraction. OTA was detected in husk insoluble fraction (4.3 µg/kg) in amounts below the maximum levels of 5 µg/kg established by the European Commission (European Commission, 2005) (Table 4). In contrast, OTA levels in husk extract were below the quantification limit (< 0.3 µg/kg). Furthermore, neither aflatoxin B1 nor enniantin B were detected in husk extract or its solid residue. García-Moraleja *et al*. (2015) reported OTA, Aflatoxin B1 and Enniantin B as the most frequent mycotoxins in coffee beverages (Garcia-Moraleja *et al*., 2015).

Table 4. Microbial and food processing contaminants.

	Husk		Parchment	CS	
	Extract	**Insoluble fraction**		**Extract**	**Insoluble fraction**
Mycotoxins (µg/Kg)					
Aflatoxin B1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Enniantin B	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Ochratoxin A	< 0.3	4.3 ± 0.5[a]	2.7 ± 0.1[a]	< 0.3	2.9 ± 0.5[a]
Acrylamide (µg/Kg)	N.D	N.D	N.D	489	< 50

N.D. non determined. Data represent means ± SD of duplicate of analysis. Different letters denote statistically significant differences between all treatments (Tuckey Test, $p < 0.05$).

The chemical composition and antioxidant capacity of coffee husk underline its potential as a food ingredient with promising health-promoting properties. For instance, coffee pulp has been proposed as a potential source of anthocyanins (pigments widely distributed in colored fruits and flowers). Murthy *et al*. (2012) reported cyanadin-3-rutinoside as the major anthocyanin in coffee pulp. Interest in anthocyanins has emerged due to their potential health benefits as antioxidant, anticarcinogenic, anti-inflammatory and hypoglycemic agents and as insulin sensitivity promoters (Lila, 2004). Coffee husk has also been used in the production of fructooligosaccharides (FOS), low caloric, non-carcinogenicity fructose oligomers which promote decreasing levels of phospholipids, triglycerides, and cholesterol. This helps gut absorption of calcium and magnesium thereby stimulating probiotic growth in the human colon (Mussatto *et al*., 2009).

Considering the extraction yield, the chemical composition of both fractions, the *in vitro* antioxidant capacity and the effect on HepG2 intracellular ROS production, we propose the fractioning of coffee husk into two novel ingredients. First, an enriched extract with potential implementation as a food preservative, natural colorant or health-promoting food ingredient, and another fraction used as a source of antioxidant dietary fiber.

3.2. Characterization of coffee parchment and its potential applications

As an extended approach to standard analytical characterization, we investigated parchment using vibrational spectroscopy as a non-invasive technique. Vibrational spectroscopy is a reliable tool to characterize structural composition in coffee (Keidel *et al*., 2010) and a rapid analytical tool for extracts and future food and health applications. In the Raman spectrum (Figure 2A), a strong, broad envelope containing C-H stretching modes was observed, possibly of CH_2-functional groups in the highest frequency region

(2700-3600 cm^{-1}). Main peaks were at 2937 and 2896 cm^{-1}. At lower frequencies a peak at 2712 cm^{-1} was identified, which is characteristic of the H-stretching modes of a O-CH_2-O group or an CH_3 umbrella overtone. Within the broad band envelope, C-H stretching modes rising also aromatic systems have the strong contribution, since they are highly Raman active. Conversely, fewer IR-bands (Figure 2B 2700-3600 cm^{-1} region) were found. At even higher frequencies, namely at 3322 cm^{-1}, we found a weak Raman-active yet strong and broad IR-active peak. This was assigned to alcohol O-H stretching. Only two IR-active peaks were identified at lower frequencies at 2854 and 2924 cm^{-1}, both related to CH_2-stretching coordinates (Supplementary Material, Table S2).

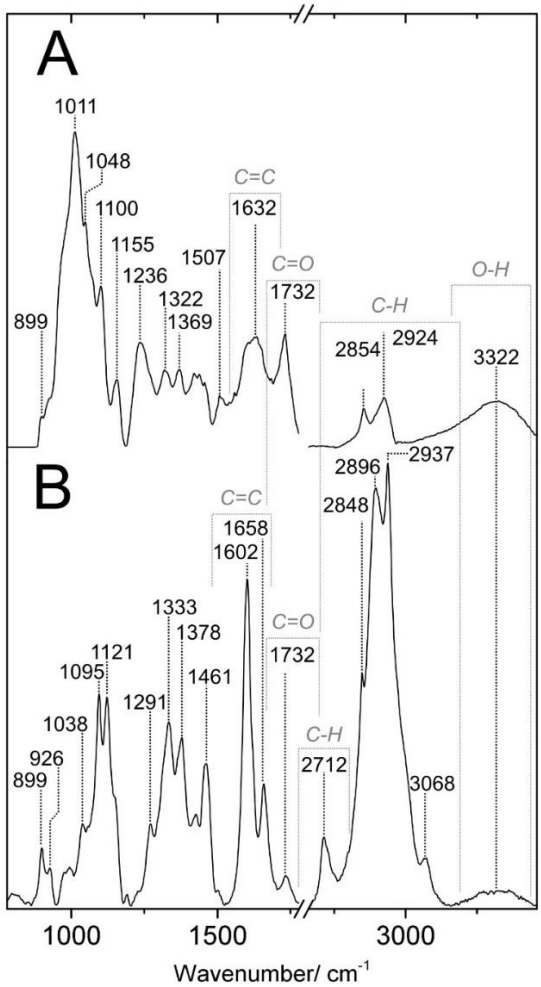


Figure 2. IR and Raman spectrum of coffee parchment. Relevant bands and vibrational modes are indicated.

At 1602 and 1658 cm^{-1}, two C=C stretching modes with strong Raman activity and negligible IR intensity were identified. In fact, this region in the IR spectrum was rather broad. As the broad peak at 1632 cm^{-1} might contain small contributions of amid-I modes, a low, yet detectable protein concentration in the parchment (*vide infra*) is assumed. The band at 1602 cm^{-1} was assigned to an aromatic ring def., whereas the peak at higher frequencies contained contributions rising from a C=C double bond, possibly in the vicinity of a benzyl (or phenyl) group. At 1732 cm^{-1} a strong IR-active and a weak Raman band associated with carbonyl C=O stretching coordinates were detected.

The further assignment mainly refers to the analysis of the parchment Raman spectrum. At lower frequencies, namely between 1000 and 1500 cm^{-1}, (in-plane) bending and deformation modes gave rise to the main contribution in this region. These allowed a further structural assignment of endocarp composition. The band at 1461 cm^{-1} was assigned to the CH_3 (or CH_2) flowering mode of methyl (methylene) coordinates, whereas the peak at 1378 cm^{-1} contained further coordinates of the CH_2, HCC, HCO and COH bending modes. Unlike double bond stretching modes, the potential energy distribution of these vibrations is less localized and includes relatively large contributions of distinct coordinates. Deformation of the O-H groups in aliphatic and aryl alcohols contribute to the bands at 1333 and 1278 cm^{-1}, respectively. A band containing glycoside symmetric ring breathing was located at 1095 cm^{-1}, whereas the peak at 1038 cm^{-1} mainly contained contributions of C-C and C-O stretching coordinates. Finally, the peak at 899 cm^{-1} was assigned to HCC and HCO bending modes. The adjacent peak at 926 cm^{-1} probably contained further angle bending modes (CHO, CCH or similar), however an unambiguous assignment was not possible. While the Raman spectrum contained very strong bending modes, only a broad and prominent envelope at 1011 cm^{-1} with band shoulders at 1048 and 1100 cm^{-1} was found. Here the highest peak and further low-frequency peaks could be assigned to C-O stretching modes of aryl alcohols, whereas the high frequency shoulders contained C-O stretching contributions of aliphatic alcohols, esters or ethers.

In agreement with physicochemical studies on coffee parchment composition, the bands at 1658 and 1602 cm^{-1} have been assigned to lignin. Conversely, cellulose did not display any bands in this region. The corresponding marker bands were observed at 899, 1095 and 1121 cm^{-1}. In the high frequency region (2700-3500 cm^{-1}), the strongest peak at 2937 cm^{-1} corresponds to lignin, whereas the adjacent peaks at 2896 and 2712 cm^{-1} correspond to the C-H stretching modes of cellulose. The O-H stretching peak has contributions from both molecules. The strong C=O stretching mode observed at 1732 cm^{-1} could not be assigned to either of the previously mentioned compounds. It could possibly reflect a

third compound group, such as hemicellulose. The observed IR and Raman activity of the C=O group (*vide supra*), can only be understood if the C=O group in discussion is conjugated, as for example in ferulated xylan. The presence of ashes, coal and dark materials give rise to strong thermal emission in the Raman spectrum. If present, the contribution is low or negligible.

Two components could be unambiguously confirmed in coffee parchment, namely lignin and cellulose. Bekalo and Reinhardt (2010) also reported the presence of these compounds in coffee parchment. Strong indications for a third species were presented. This combined spectroscopic approach provides complementary information. However, further studies of different parchment varieties, vibrational spectra of isolated compounds as well as extended chemometric analysis are required. It is interesting to note that the obtained results provide the first vibrational spectroscopy (IR and Raman) characterization of coffee by-products.

Parchment showed the lowest extraction yield (2.3 %). This extract (Figure 1b) presented low levels of 5-CQA (6.1 mg/g); however, it had the highest caffeine content (58.2 mg/g) ($p < 0.05$). No bibliographical references have been found regarding caffeine and CGA content in aqueous extracts of coffee parchment. As shown in Table 1, parchment extract had the greatest antioxidant capacity ($p < 0.05$) of all the samples analyzed, followed by CS extracts. Parchment and Robusta CS extract showed the highest TPC and TFC ($p < 0.05$). Table 2 includes data on the effect of parchment extract on cell viability and intracellular ROS formation. At concentrations of 0.1 and 1 mg/ml cell viability was not significantly ($p > 0.05$) altered, while 10 and 20 mg/ml resulted cytotoxic. Pre-treatment of cells with parchment extract for 24 hours (1 mg/ml) significantly reduced ($p < 0.05$) ROS levels compared to non-treated cells and cells treated with tBOOH. Parchment extract had a greater antioxidant effect on HepG2 cells compared to husk extract. Results are in line with those corresponding to TAC and TPC. Parchment extract presented the highest values for these parameters among the studied samples. To the best of our knowledge, no studies have been published on the effect of coffee parchment on cell viability and intracellular ROS formation.

The insoluble fraction of coffee parchment obtained after extraction presented the highest amount of TDF ($p < 0.05$) (92.6 %) mainly composed of IDF (92.1 %) (Table 3). Protein content in the parchment insoluble fraction was 3.1%, which was the lowest value compared to the rest of the samples. Our results showed that parchment insoluble fraction also displayed the lowest ($p < 0.05$) lipid content (0.3 %). To date, no other data

on protein and lipid content of coffee parchment have been published. Furthermore, it also had the lowest antioxidant capacity (4.1 mg CGA/g).

Considering the food safety of raw parchment, none of the studied pesticides were detected in this sample. Aflatoxin B1 and enniantin B were not present in parchment (Table 4). However, OTA was detected at 2.7 µg/kg, which is under the limit established by the European Commission (5 µg/kg). Therefore, the raw material should be thermally stabilized to avoid microbial contamination and the production of mycotoxins during its storage, to increase the shelf-life of the sample and to ensure its safe use as a food ingredient for human beings.

Until now, applications for coffee parchment as a food ingredient remain largely unexplored. Despite the promising chemical composition of aqueous parchment extract and its high *in vitro* antioxidant capacity, we suggest that coffee parchment should be used as it is after a simple thermal stabilization, since extraction yields are rather poor. New applications of coffee parchment in the food industry as a natural source of antioxidant dietary fiber should be considered to revalue this by-product.

3.3. Characterization of coffee silverskin and its potential applications

The extraction yield of water-soluble compounds from CS (Figure 1c) was similar for Arabica and Robusta species. As CS is in direct contact with the coffee seeds, CS extract presented the highest content of 5-CQA (9.4 mg/g and 21.3 mg/g, for Arabica and Robusta species, respectively). The concentration ratio of caffeine/5-CQA was 2.5 in both the Arabica and Robusta CS extract. However, values of both compounds were lower in the Arabica CS extract (Table 1). The obtained caffeine content was higher than that reported by other authors (Bresciani *et al*., 2014; Napolitano *et al*., 2007). In agreement with our results, Mesías *et al*. (2014) found that 5-CQA and caffeine content were significantly higher in Robusta than in Arabica CS extract.

EFSA reported a safety level for daily caffeine consumption of 400 mg for the general population and 200 mg for lactating women. There is currently not enough information available to determine a safe level of caffeine intake for children (European Food Safety Authority (EFSA), 2015). Caffeine also presents beneficial health effects due to its stimulating properties (Nehlig, 2016). Arabica and Robusta CS extracts could be a natural source of sustainable caffeine. So far, published results suggest that caffeine content should not be considered as a safety concern in the application of coffee by-products as food ingredients (Garcia-Serna *et al*., 2014; Martinez-Saez *et al*., 2014). However, more conclusive studies are required.

Robusta CS extract presented significantly higher amounts of TPC and TFC than Arabica CS extract ($p < 0.05$), and TPC and TFC concentration was significantly higher in these two species than in husk extract ($p < 0.05$) (Table 1). In this case, the TPC value obtained for CS extracts was in the same order of magnitude as the values obtained by Mesías *et al*. (2014). The antioxidant capacity values of the different CS extracts did not differ significantly ($p > 0.05$). There is usually a greater proportion of antioxidants in Robusta CS than in Arabica CS (Mesías *et al*., 2014; Napolitano *et al*., 2007). Other authors have also confirmed the antioxidant character and high phenolic and flavonoid content in this sample (Borrelli *et al*., 2004; Rodrigues *et al*., 2015a). Although TAC was significantly greater in parchment extract ($p < 0.05$), parchment extraction yield is low. Therefore, CS could be a more suitable source of bioactive compounds with a higher antioxidant capacity. The application of CS as a natural source of antioxidants has been previously described (del Castillo *et al*., 2013; Garcia-Serna *et al*., 2014; Iriondo-DeHond *et al*., 2016; Mesías *et al*., 2014; Rodrigues *et al*., 2015).

Since Arabica coffee represents around 70 % of global production (Bunn *et al*., 2015), this species was used in the *in vitro* studies. Arabica CS extract was not cytotoxic in HepG2 cells at none of the tested concentrations. For doses of 10 and 20 mg/ml of Arabica CS extract, cell viability increased significantly ($p < 0.05$) (Table 2). The non-cytotoxic character of CS has also been studied in other cell lines (Iriondo-DeHond *et al*., 2016; Iriondo-DeHond *et al*., 2017; Rodrigues *et al*., 2015). Arabica CS extract was more effective in the reduction of induced ROS than husk extract, and as effective as parchment extract. This is in agreement with their corresponding antioxidant capacity studied *in vitro*. The protective effect of CS in the reduction of induced ROS formation has been reported for other cell lines (Fernandez-Gomez *et al*., 2016; Iriondo-DeHond *et al*., 2016).

The TDF content in the remaining CS solid insoluble fraction after the aqueous extraction process was similar for Arabica and Robusta varieties (Table 3). Values obtained for CS dietary fiber are similar to those previously described (Ballesteros *et al*., 2014; Borrelli *et al*., 2004; Garcia-Serna *et al*., 2014). Most of the dietary fiber found in the sample was insoluble, and values of SDF were lower than those recently described (Esquivel & Jiménez, 2012; Napolitano *et al*., 2007). In agreement with previous results (Ballesteros *et al*., 2014; Borrelli *et al*., 2004; Pourfarzad *et al*., 2013), the highest protein content corresponded to Robusta CS (19 %). Lipid content of the insoluble residues of CS was 2.6 % and 3.6 % for Arabica and Robusta, respectively, in the same range as that previously described by Ballesteros *et al*. (2014) and Borrelli *et al*. (2004).

Food safety of Arabica CS was validated by the absence of pesticides, aflatoxin B1 and enniantin B (Table 4). OTA was present in the insoluble fraction of this sample (2.9 µg/kg) in a concentration below the limit established by the European Commission (5 µg/kg) and was not detected in CS extract. Other authors have described the presence of OTA in CS (Toschi *et al*., 2014). The analysis of this contaminant is mandatory to ensure the safety of CS extract as a food ingredient. Since CS is the only by-product generated during the roasting process of coffee beans, acrylamide content was also studied in this sample. Levels of 489 µg/kg of this water-soluble compound were found in CS extract. This value is between the values established by the European Commission for roasted coffee (400 µg/kg) and instant coffee (850 µg/kg) (European Commission, 2017). In contrast, CS insoluble fraction presented levels < 50 µg/kg. Mitigation of acrylamide in CS can be achieved following the instructions recommended for roasted and instant coffees by the European Commission.

Arabica CS, CS extract and the insoluble fraction recovered from the water extraction process have been implemented to improve the formulation of biscuits. In this study, CS was used as a natural colorant and as a source of fiber (Garcia-Serna *et al*., 2014). CS has also been used to improve the quality, shelf-life, sensory qualities and appearance of Barbari bread (Pourfarzad *et al*., 2013). Other authors proposed the use of CS as a source of prebiotics (Borrelli *et al*., 2004; Jiménez-Zamora *et al*., 2015; Mussatto *et al*., 2013). In addition, CS has been used in combination with roasted coffee and cocoa powder to obtain innovative coffee blends that were rich in bioactive compounds, such as CGAs, trigonelline, theobromine, and caffeine (Ribeiro *et al*., 2014). Other novel antioxidant beverages based on raw CS and CS extract from Arabica and Robusta species have been developed to study their inhibitory effect on fat accumulation using *Caenorhabditis elegans* as an *in vivo* model (Martinez-Saez *et al*., 2014). According to our results, improved fractioning of CS will yield a superior aqueous extract rich in bioactive compounds with the potential to reduce diabetes and obesity, among other conditions. Similarly, the potential of coffee husk extract to reduce the risk of chronic diseases should also be studied.

Considering the extraction yield and chemical characterization of each by-product fraction, we suggest single-fraction separation and development of two new multifunctional ingredients. As proposed for coffee husk, CS is a suitable source for an enriched extract which could be implemented as a food ingredient with antioxidant and antidiabetic properties (del Castillo *et al*., 2016). Furthermore, the insoluble fraction can be used as a source of antioxidant dietary fiber.

3.4. Food toxicity of novel ingredients

Single oral administration of the three validated coffee by-products (raw parchment and husk and Arabica CS extracts) at a dose of 2000 mg/kg b.w. showed no visible signs of toxicity, abnormal behavior or mortality. Relative organ weights are shown in Table S3 (Supplementary Material). No significant differences ($p > 0.05$) were found among groups. Intake of an acute dose of ingredients (2000 mg/kg b.w) did not cause significant changes in histological parameters of vital organs. Livers of the control and treated rats showing normal hepatic lobules, polyhedral hepatocytes with central vesicular nuclei and eosinophilic granular cytoplasm are shown in Figure 3a and 3b, respectively. Similarly, the control and treated kidney sections showed a normal histological picture composed of renal corpuscles, which appear as rounded structures, and glomeruli are surrounded by narrow Bowman's spaces and cortical tubules with a small number of distal convoluted tubules and collecting tubules (Figure 3c and 3d).

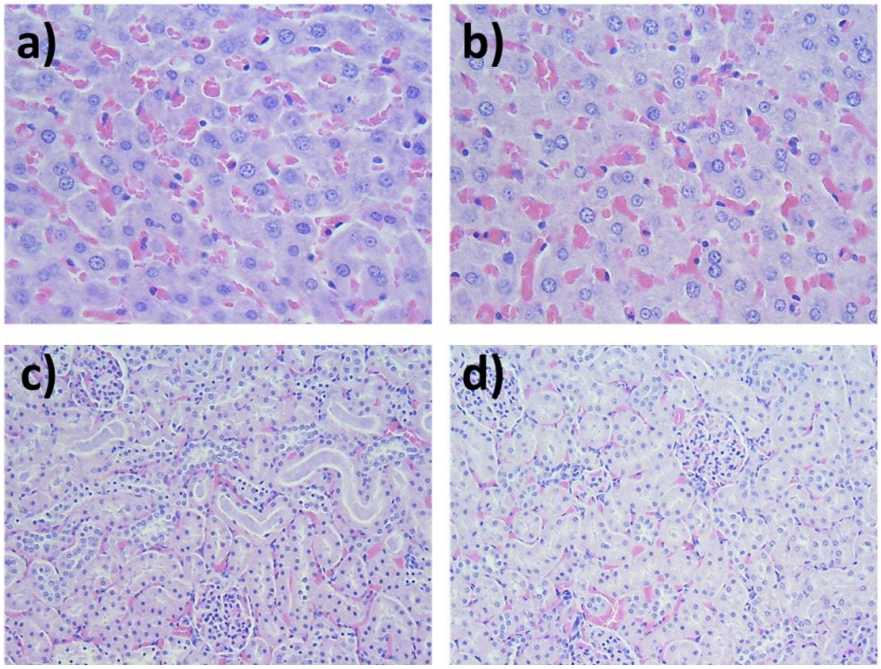


Figure 3. Sections of liver (a,b) and kidney (c,d) of female rats from control group (a, c), and the group treated with husk extract (b, d), showing a normal architecture in both tissues (H&E x400).

To the best of our knowledge, very few studies regarding the presence of contaminants in coffee by-products have been previously published. No lethal effects were observed in rats treated with 2000 mg/kg b.w. by oral administration. Results are supported by

data on contaminants. As previously discussed, pesticides, aflatoxin B1 and enniantin B were not found in the coffee by-products; OTA was detected in raw parchment and in the insoluble fraction of husk and CS in concentrations lower than 5 µg/kg; acrylamide was only detected in CS and its derivatives in lower or similar concentrations to those found in roasted coffee (400 µg/kg) (European Commission, 2017). Caffeine concentrations can be considered safe and were lower than concentrations reported for coffee beverages (European Food Safety Authority (EFSA), 2015).

4. Conclusions

This study provided data for the validation of CS and coffee husk as two novel safe food ingredients: an enriched extract with multifunctional properties and an insoluble fraction composed of dietary fiber of different nature (soluble and insoluble). The fractioning procedure is a sustainable, low-cost process which could be easily performed by the coffee industry without the production of new waste. We also propose that coffee parchment be used as a single food ingredient composed of antioxidant insoluble dietary fiber after a thermal stabilization process. Thus, coffee by-products can be converted into promising safe health-promoting food ingredients, providing sustainable economic and environmental benefits. Analysis of contaminants is essential to achieve this goal.

Acknowledgements

The SUSCOFFEE Project (AGL2014-57239-R): "Sustainable coffee production and consumption: Valorization of coffee waste into food ingredients" funded this work. A. Iriondo-DeHond is a fellow of the FPI predoctoral program of MINECO (BES-2015-072191). The authors thank Dr. Miguel Herrero for his help with the UPLC-MS/MS analysis.

References

Açar, Ö. C., Gökmen, V., Pellegrini, N., & Fogliano, V. (2009). Direct evaluation of the total antioxidant capacity of raw and roasted pulses, nuts and seeds. *European Food Research and Technology*, *229*(6), 961–969. https://doi.org/10.1007/s00217-009-1131-z.

Adebajo, M. O., Frost, R. L., Kloprogge, J. T., & Kokot, S. (2006). Raman spectroscopic investigation of acetylation of raw cotton. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *64*(2), 448–453. https://doi.org/10.1016/J.SAA.2005.07.045.

Afsah-Hejri, L., Jinap, S., Hajeb, P., Radu, S., & Shakibazadeh, S. (2013). A Review on Mycotoxins in Food and Feed: Malaysia Case Study. *Comprehensive Reviews in Food Science and Food Safety*, *12*(6), 629–651. https://doi.org/10.1111/1541-4337.12029.

Bakondi, E., Gönczi, M., Szabó, É., Bai, P., Pacher, P., Gergely, P., … Virág, L. (2003). Role of intracellular calcium mobilization and cell-density-dependent signaling in oxidative-stress-induced cytotoxicity in HaCaT keratinocytes. *Journal of Investigative Dermatology*, *121*(1), 88–95. https://doi.org/10.1046/j.1523-1747.2003.12329.x.

Ballesteros, L. F., Teixeira, J. a., & Mussatto, S. I. (2014). Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. *Food and Bioprocess Technology*, *7*(12), 3493–3503. https://doi.org/10.1007/s11947-014-1349-z.

Bekalo, S. A., & Reinhardt, H.-W. W. (2010). Fibers of coffee husk and hulls for the production of particleboard. *Materials and Structures*, *43*(8), 1049–1060. https://doi.org/DOI 10.1617/s11527-009-9565-0.

Belitz, H.-D., Grosch, W., & Schieberle, P. (2009). Coffee, tea, cocoa. In: *Food Chemistry*. Eds. Belitz, H.-D., Grosch, W. and Schieberle, P., 4th edition, Springer, Leipzig (pp. 938–951).

Borrelli, R. C., Esposito, F., Napolitano, A., Ritieni, A., & Fogliano, V. (2004). Characterization of a new potential functional ingredient: coffee silverskin. *Journal of Agricultural and Food Chemistry*, *52*(5), 1338–1343. https://doi.org/10.1021/jf034974x.

Borrelli, R. C., Visconti, A., Mennella, C., Anese, M., & Fogliano, V. (2002). Chemical characterization and antioxidant properties of coffee melanoidins. *Journal of Agricultural and Food Chemistry*, *50*(22), 6527–33. https://doi.org/10.1021/jf025686o.

Brand, D., Pandey, a., Rodriguez-Leon, J. a., Roussos, S., Brand, I., & Soccol, C. R. (2001). Packed bed column fermenter and kinetic modeling for upgrading the nutritional quality of coffee husk in solid-state fermentation. *Biotechnology Progress*, *17*(6), 1065–1070. https://doi.org/10.1021/bp010112.

Bresciani, L., Calani, L., Bruni, R., Brighenti, F., & Del Rio, D. (2014). Phenolic composition, caffeine content and antioxidant capacity of coffee silverskin. *Food Research International*, *61*, 196–201. https://doi.org/10.1016/j.foodres.2013.10.047.

Bruntha Devi, P., Vijayabharathi, R., Sathyabama, S., Gurusiddappa Malleshi, N., & Brindha Priyadarisini, V. (2011). Health benefits of finger millet (*Eleusine coracana* L.) polyphenols and dietary fiber: a review. https://doi.org/10.1007/s13197-011-0584-9.

Bunn, C., Läderach, P., Ovalle Rivera, O., & Kirschke, D. (2015). A bitter cup: climate change profile of global production of Arabica and Robusta coffee. *Climatic Change*, *129*(1–2), 89–101. https://doi.org/10.1007/s10584-014-1306-x.

Chanakya, H. N., & De Alwis, A. A. P. (2004). Environmental Issues and Management in Primary Coffee Processing. *Process Safety and Environmental Protection*, *82*(4), 291–300. https://doi.org/10.1205/095758204323162319.

Contini, M., Baccelloni, S., Massantini, R., & Anelli, G. (2008). Extraction of natural antioxidants from hazelnut (Corylus avellana L.) shell and skin wastes by long maceration at room temperature. *Food Chemistry*, *110*(3), 659–669. https://doi.org/10.1016/j.foodchem.2008.02.060.

del Castillo, M. D., Fernandez-Gomez, B., Martinez-Saez, N., Iriondo-DeHond, A., Martirosyan, D. M., & Mesa, M. D. (2016). Coffee silverskin extract for aging and chronic diseases. In D. M. Martirosyan (Ed.), *Functional Foods In Health And Disease*. CreateSpace Independent Publishing Platform.

del Castillo, M. D., Fernandez-Gomez, B., Martinez-Saez, N., Iriondo-DeHond, A., & Mesa, M. D. (2018). Coffee By-Products. In A. Farah (Ed.), *Coffee: Chemistry, Quality and Health Implications.* Oxfordshire: Royal Society of Chemistry.

del Castillo, M. D., Ibañez, M. E., Amigo, M., Herrero, M., Plaza del Moral, M., & Ullate, M. (2013, January). *Application of products of coffee silverskin in anti-ageing cosmetics and functional food*. WO 2013/004873.

del Castillo, M. D., Iriondo-DeHond, A., Fernandez-Gomez, B., Martinez-Saez, N., Iriondo-DeHond, M., & Zhou, J.-R. (2017). Applications of recovered compounds in food products. In C. Galanakis (Ed.), *Handbook of Coffee Processing By-Products: Sustainable Applications (First Edit).* Academic Press-Elsevier.

Esquivel, P., & Jiménez, V. M. (2012). Functional properties of coffee and coffee by-products. *Food Research International*, *46*(2), 488–495. https://doi.org/10.1016/j.foodres.2011.05.028.

European Commission. (2005). COMMISSION REGULATION (EC) No 123/2005 of 26 January 2005 amending Regulation (EC) No 466/2001 as regards ochratoxin A. *Official Journal of the European Union*, (123), 2004–2006. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R0123&qid=1478028660364&from=ES.

European Commission. (2014). Proposal for a directive of the European parliament and of the council amending Directives 2008/98/EC on waste, 94/62/EC on packaging and packaging waste, 1999/31/EC on the landfill of waste, 2000/53/EC on end-of-life vehicles, 2006/66/EC on batteries and. *Official Journal of the European Union*, *0201*, 1–23. https://doi.org/10.1017/CBO9781107415324.004.

European Commission. (2017). COMMISSION REGULATION (EU) 2017/2158 of 20 November 2017 establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food. *Official Journal of the European Union*, *204*, 24–44. https://doi.org/http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_285/l_28520031101en00330037.pdf.

European Food Safety Authority (EFSA) (2015). Scientific Opinion on the safety of caffeine. *EFSA Journal*, *13*(5), 1–21. https://doi.org/10.2903/j.efsa.2015.4102.

European Food Safety Authority (EFSA) (2016). Chemicals in food 2016. *Parma*. https://doi.org/10.2805/359743.

Fernandez-Gomez, B., Lezama, A., Amigo-Benavent, M., Ullate, M., Herrero, M., Martín, M. Á., … del Castillo, M. D. (2016). Insights on the health benefits of the bioactive compounds of coffee silverskin extract. *Journal of Functional Foods*, *25*, 197–207. https://doi.org/10.1016/j.jff.2016.06.001.

Fernandez-Gomez, B., Ramos, S., Goya, L., Mesa, M. D., del Castillo, M. D., & Martín, M. Á. (2016). Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells. *Food Research International. 89,* 1015-1022. https://doi.org/.doi.org/10.1016/j.foodres.2016.03.006

Folch, J., Lees, M., & Sloane Stanley, G. (1987). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, *226*, 497–509. http://dx.doi.org/10.1016/j.ultrasmedbio.2011.03.005.

Gamboa-Gaitán, M. Á. (2012). Presencia de aspergillus y otros simbiontes fúngicos en granos de café procedentes de Colombia, *17*(1), 39–50. Retrieved from https://www.scopus.com/record/display.uri?eid=2-s2.0-84861470773&origin=inward&txGid=6c7e709f19507c1f886634e01289d794.

Garcia-Moraleja, A., Font, G., Manes, J., & Ferrer, E. (2015). Analysis of mycotoxins in coffee and risk assessment in Spanish adolescents and adults. *Food and Chemical Toxicology*, *86*, 225–233. https://doi.org/10.1016/j.fct.2015.10.014.

Garcia-Serna, E., Martinez-Saez, N., Mesias, M., Morales, F. J., & del Castillo, M. D. (2014). Use of Coffee Silverskin and Stevia to Improve the Formulation of Biscuits. *Polish Journal of Food and Nutrition Sciences*, *64*(4), 243–251. https://doi.org/10.2478/pjfns-2013-0024.

Gierlinger, N., Keplinger, T., & Harrington, M. (2012). Imaging of plant cell walls by confocal Raman microscopy. *Nature Protocols*, *7*(9), 1694–1708. https://doi.org/10.1038/nprot.2012.092.

Gierlinger, N., Keplinger, T., Harrington, M., & Schwanninger, M. (2013). Raman Imaging of Lignocellulosic Feedstock. In *Cellulose - Biomass Conversion*. InTech. https://doi.org/10.5772/50878.

Gomes, A., Fernandes, E., & Lima, J. L. F. C. (2005). Fluorescence probes used for detection of reactive oxygen species. *Journal of Biochemical and Biophysical Methods, 65*(2–3), 45–80. https://doi.org/10.1016/j.jbbm.2005.10.003.

Gouvea, B. M., Torres, C., Franca, A. S., Oliveira, L. S., & Oliveira, E. S. (2009). Feasibility of ethanol production from coffee husks. *Biotechnology Letters, 31*(9), 1315–1319. https://doi.org/10.1007/s10529-009-0023-4.

Iriondo-DeHond, A., Haza, A. I., Ávalos, A., del Castillo, M. D., & Morales, P. (2017). Validation of coffee silverskin extract as a food ingredient by the analysis of cytotoxicity and genotoxicity. *Food Research International, 100*, 791–797. https://doi.org/10.1016/j.foodres.2017.08.012.

Iriondo-DeHond, A., Martorell, P., Genovés, S., Ramón, D., Stamatakis, K., Fresno, M., … del Castillo, M. D. (2016). Coffee silverskin extract protects against accelerated aging caused by oxidative agents. *Molecules, 21*(6), 1–14. https://doi.org/10.3390/molecules21060721.

Jiménez-Zamora, A., Pastoriza, S., & Rufián-Henares, J. a. (2015). Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties. *LWT - Food Science and Technology, 61*(1), 12–18. https://doi.org/10.1016/j.lwt.2014.11.031.

Keidel, A., von Stetten, D., Rodrigues, C., Máguas, C., & Hildebrandt, P. (2010). Discrimination of Green Arabica and Robusta Coffee Beans by Raman Spectroscopy. *Journal of Agricultural and Food Chemistry, 58*(21), 11187–11192. https://doi.org/10.1021/jf101999c.

Lila, M. A. (2004). Anthocyanins and Human Health: An In Vitro Investigative Approach. *Journal of Biomedicine & Biotechnology, 2004*(5), 306–313. https://doi.org/10.1155/S111072430440401X.

Martinez-Saez, N., Ullate, M., Martin-Cabrejas, M. a., Martorell, P., Genovés, S., Ramon, D., & Del Castillo, M. D. (2014). A novel antioxidant beverage for body weight control based on coffee silverskin. *Food Chemistry, 150*, 227–234. https://doi.org/10.1016/j.foodchem.2013.10.100.

Mesías, M., Navarro, M., Martínez-Saez, N., Ullate, M., del Castillo, M. D., & Morales, F. J. (2014). Antiglycative and carbonyl trapping properties of the water soluble fraction of coffee silverskin. *Food Research International, 62*, 1120–1126. https://doi.org/10.1016/j.foodres.2014.05.058.

Murthy, P. S., Manjunatha, M., Sulochannama, G., & Madhava Naidu, M. (2012). Extraction, Characterization and Bioactivity of Coffee Anthocyanins. *European Journal of Biological Sciences, 4*(1), 13–19. https://doi.org/10.5829/idosi.ejbs.2012.4.1.6149.

Murthy, P. S., & Naidu, M. M. (2012). Recovery of Phenolic Antioxidants and Functional Compounds from Coffee Industry By-Products. *Food and Bioprocess Technology*, *5*(3), 897–903. https://doi.org/10.1007/s11947-010-0363-z.

Mussatto, S. I., Aguilar, C. N., Rodrigues, L. R., & Teixeira, J. A. (2009). Fructooligosaccharides and β-fructofuranosidase production by *Aspergillus japonicus* immobilized on lignocellulosic materials. *Journal of Molecular Catalysis B: Enzymatic*, *59*(1–3), 76–81. https://doi.org/10.1016/j.molcatb.2009.01.005.

Mussatto, S. I., Ballesteros, L. F., Martins, S., Maltos, D. A. F., Aguilar, C. N., & Teixeira, J. A. (2013). Maximization of Fructooligosaccharides and β-Fructofuranosidase Production by Aspergillus japonicus under Solid-State Fermentation Conditions. *Food and Bioprocess Technology*, *6*(8), 2128–2134. https://doi.org/10.1007/s11947-012-0873-y.

Nabais, J. M. V., Nunes, P., Carrott, P. J. M., Ribeiro Carrott, M. M. L., García, a. M., & Díaz-Díez, M. a. (2008). Production of activated carbons from coffee endocarp by CO_2 and steam activation. *Fuel Processing Technology*, *89*(3), 262–268. https://doi.org/10.1016/j.fuproc.2007.11.030.

Napolitano, A., Fogliano, V., Tafuri, A., & Ritieni, A. (2007). Natural occurrence of ochratoxin A and antioxidant activities of green and roasted coffees and corresponding byproducts. *Journal of Agricultural and Food Chemistry*, *55*(25), 10499–10504. https://doi.org/10.1021/jf071959.

Navya, P. N., & Pushpa, S. M. (2013). Production, statistical optimization and application of endoglucanase from Rhizopus stolonifer utilizing coffee husk. *Bioprocess and Biosystems Engineering*, *36*(8), 1115–1123. https://doi.org/10.1007/s00449-012-0865-3.

Nehlig, A. (2016). Effects of coffee/caffeine on brain health and disease: What should I tell my patients? *Practical Neurology*, *16*(2), 89–95. https://doi.org/10.1136/practneurol-2015-001162.

Oki, T., Nagai, S., Yoshinaga, M., Nishiba, Y., & Suda, I. (2006). Contribution of b-Carotene to Radical Scavenging Capacity Varies among Orange-fleshed Sweet Potato Cultivars. *Food Science and Technology Research*, *12*, 156–160. https://doi.org/10.3136/fstr.12.156.

Oecd/Ocde (2008). Acute oral toxicity–up-and-down-procedure (UDP). *OECD Guidelines for the Testing of Chemicals*, *425*, 1–27. https://dx.doi.org/10.1787/9789264071049-en.

Onyango, C., Noetzold, H., Bley, T., & Henle, T. (2004). Proximate composition and digestibility of fermented and extruded uji from maize–finger millet blend. *LWT - Food Science and Technology*, *37*(8), 827–832. https://doi.org/10.1016/J.LWT.2004.03.008.

Pandey, A., Soccol, C. R., Nigam, P., Brand, D., Mohan, R., & Roussos, S. (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochemical Engineering Journal*, *6*(2), 153–162. https://doi.org/10.1016/S1369-703X(00)00084-X.

Pourfarzad, A., Mahdavian-Mehr, H., & Sedaghat, N. (2013). Coffee silverskin as a source of dietary fiber in bread-making: Optimization of chemical treatment using response surface methodology. *LWT - Food Science and Technology*, *50*(2), 599–606. https://doi.org/10.1016/j.lwt.2012.08.001.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3.

Ribeiro, V. S., Leitão, A. E., Ramalho, J. C., & Lidon, F. C. (2014). Chemical characterization and antioxidant properties of a new coffee blend with cocoa, coffee silverskin and green coffee minimally processed. *Food Research International*, *61*, 39–47. https://doi.org/10.1016/j.foodres.2014.05.003.

Rodrigues, F., Gaspar, C., Palmeira-de-Oliveira, A., Sarmento, B., Helena Amaral, M., & P P Oliveira, M. B. (2015). Application of Coffee Silverskin in cosmetic formulations: physical/antioxidant stability studies and cytotoxicity effects. *Drug Development and Industrial Pharmacy*, *00*(00), 1–8. https://doi.org/10.3109/03639045.2015.1035279.

Rodrigues, F., Palmeira-de-Oliveira, A., das Neves, J., Sarmento, B., Amaral, M. H., & Oliveira, M. B. P. P. (2015a). Coffee silverskin: a possible valuable cosmetic ingredient. *Pharmaceutical Biology*, *53*(3), 386–94. https://doi.org/10.3109/13880209.2014.922589.

Rodrigues, F., Palmeira-de-Oliveira, A., das Neves, J., Sarmento, B., Amaral, M. H., & Oliveira, M. B. P. P. (2015b). Coffee silverskin: a possible valuable cosmetic ingredient. *Pharmaceutical Biology*, *53*(3), 386–94. https://doi.org/10.3109/13880209.2014.922589.

Singh, R., & Geetanjali. (2013). Nutraceuticals: Promising Health Product. *International Research Journal of Medical Sciences*, *1*(1), 14–17. Retrieved from http://www.isca.in.

Toschi, T. G., Cardenia, V., Bonaga, G., Mandrioli, M., & Rodriguez-estrada, M. T. (2014). Coffee Silverskin : Characterization, Possible Uses, and Safety Aspects. *Journal of Agricultural and Food Chemistry*, *62*, 10836–10844.

Ulijasz, A. T., Cornilescu, G., von Stetten, D., Cornilescu, C., Velazquez Escobar, F., Zhang, J., … Vierstra, R. D. (2009). Cyanochromes Are Blue/Green Light Photoreversible Photoreceptors Defined by a Stable Double Cysteine Linkage to a Phycoviolobilin-type

Chromophore. *Journal of Biological Chemistry, 284*(43), 29757–29772. https://doi.org/10.1074/jbc.M109.038513.

Xu, B. J., & Chang, S. K. C. (2007). A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *Journal of Food Science, 72*(2). https://doi.org/10.1111/j.1750-3841.2006.00260.x.

Table S1. Automatic search parameters for the pesticide mixture analyzed in GC-MS (Sim mode). (*) Second injection.

Pesticide	Molecular mass (m/z)	Molecular ions (m/z)	Time window (min)
HCH-α	290.8	71, 253, 255	5.0 – 22.0
HCH-β	290.8	71, 253, 255	5.0 – 22.0
HCH-γ	290.8	71, 253, 255	5.0 – 22.0
HCH-δ	290.8	71, 253, 255	5.0 – 22.0
Heptachlor	373.3	266, 298, 300	22.0 - 24.5
Aldrin	364.9	235, 237, 330	22.0 - 24.5
Hexachlorbenzene	284.7	250, 284, 286	22.0 - 24.5
Endosulfan-α	406.9	237, 342, 372, 408	21.0 - 25.0(*)
Endosulfan-β	406.9	242, 372, 406	21.0 - 25.0(*)
Chlorpyrifos methyl	322.5	141, 214, 285	25.0 - 30.0(*)
Parathion methyl	263.2	141.154, 263	25.0 - 30.0(*)
Parathion ethyl	391.2	154, 169, 291	25.0 - 30.0(*)
Chlorpyrifos ethyl	350.6	169, 214, 313, 315	25.0 - 30.0(*)
Dieldrin	380.9	237, 346, 380	26.0 - 29.0
Endrin	380.9	346, 380	26.0 - 29.0
4,4-DDE	326.0	281, 316, 318	26.0 - 29.0
2,4-DDD	320.0	246, 248	26.0 - 29.0
44DDT	354.4	71,73, 281	29.0 - 31.0
24DDE	318.0	246, 248, 318	29.0 - 31.0
Mirex	545.5	368, 402, 435	31.0 - 40.0

Table S2. Vibrational assignment of *Coffea arabica* parchment.

Freq[1]	IR[2]	Ra[2]	Vibrational Assignment[3]	Compound[4]
3322	m	vw	ν(O-H) stretching hydroxyl groups	
3068	-	w	ν(C-H) arom. i. p. stretching	
2937	-	vs	ν(C-H) CH_2 ip. stretching	lignin
2924	mw	-	ν(C-H) CH_3 ketones	
2896	-	s	ν(C-H) oop asym str	
2854	mw	-	ν(C-H) oop asym str.	
2848	-	m	ν(C-H) oop asym str	
2712	-	w	Overtone 1378 cm-1 δ(CH_3) umbrella	
1732	m	vw	C=O stretching	hemicelluose
1658	-	m	C=C double bond stretch, adjacent to a conj. ring	lignin
1632	m	-	Amid-I most likely	protein/peptides
1602	-	s	arom. ring def.	lignin
1461	-	m	CH_2 scissoring	
1378	-	m	CH_3 umbrella	
1369	mw	-	C-H (CH_3) def. mode	
1333	-	m	ω(CH_2), δ(HCC), δ(HCO), δ(COH)	
1291	-	w	ω(CH_2), δ(HCC), δ(HCO), δ(COH)	
1236	mw	-	τ(CH2), δ(HCC), δ(HCO), δ(COH)	
1155	mw	-	γ (O-CH_3)	lignin
1121	-	m	ν(C-O-C)	cellulose
1100	m	-	ν(C-O) sat. sec. alcohol	cellulose
1095	-	m	ν(C-O-C)	cellulose
1048	w	-	ν(C-C), ν(C-O)	
1038	-	w	ν(C-C), ν(C-O)	
1011	vs	-	δ(O-CH_3), ip ring def.	
926	-	w	arom. def. mode, δ (CH_3)	lignin
899	w	w	δ(C(1)H(β))	cellulose

[1]Absolute (IR) and relative (Raman) vibrational frequencies are given in cm^{-1}.
[2]IR and Raman intensity are given relatively to the strongest peak in each spectrum.
[3]Vibrational assignment based on given references.
[4]According to vibrational assignment.

Table S3. Relative organ weights in female rats included in controls and in groups exposed to limit dose of 2000 mg/kg b.w.

Organs	Water (control)	Corn oil (control)	Husk extract	Parchment	CS extract
Hearth	0.388±0.045	0.381±0.033	0.311±0.041	0.298±0.036	0.314±0.038
Lungs	0.798±0.089	0.816±0.105	0.789±0.099	0.805±0.072	0.718±0.099
Liver	4.777±0.301	4.845±0.246	4.293±0.198	4.579±0.224	4.726±0.186
Kidneys	0.954±0.166	1.089±0.153	0.912±0.133	0.850±0.113	0.898±0.099
Spleen	0.199±0.068	0.208±0.042	0.183±0.035	0.179±0.021	0.180±0.034
Thymus	0.200±0.055	0.202±0.029	0.193±0.055	0.179±0.011	0.180±0.026
Adrenal glands	0.070±0.007	0.054±0.008	0.069±0.002	0.055±0.005	0.065±0.001
Uterus	1.011±0.122	1.070±0.102	0.979±0.111	0.926±0.105	0.963±0.156
Brain	0.892±0.101	0.925±0.099	0.979±0.110	0.861±0.201	0.818±0.187

Values represent means ± SD (n = 5). No significant differences were detected among groups (Tukey test, $p < 0.05$).

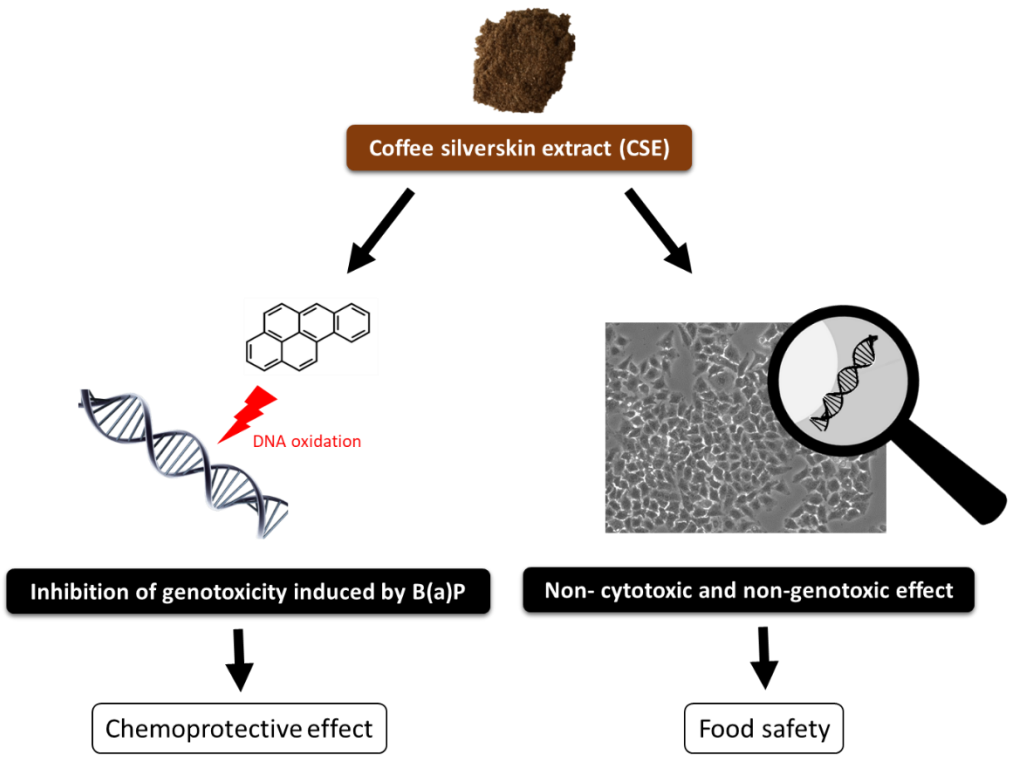
CHAPTER 2

This chapter aims to provide new knowledge for the validation of the novel ingredient obtained from the coffee roasting by-product (coffee silverskin extract, CSE):

- In Study 2, the cytotoxicity and genotoxicity and the preventive potential against induced oxidative DNA damage of CSE were studied.
- In Study 3, the nutritional value of CSE, its safety and effects of key biological functions were assessed *in vivo*.

Study 2: Validation of coffee silverskin extract as a food ingredient by the analysis of cytotoxicity and genotoxicity

Iriondo-DeHond, A., Haza, A. I., Ávalos, A., del Castillo, M. D., Morales, P. 2017. Validation of coffee silverskin extract as a food ingredient by the analysis of cytotoxicity and genotoxicity. *Food Research International*, 100, 791–797. Available at: https://doi.org/10.1016/j.foodres.2017.08.012



Validation of coffee silverskin extract as a food ingredient by the analysis of cytotoxicity and genotoxicity

Amaia Iriondo-DeHond [1], Ana I. Haza [2], Alicia Ávalos [2], María Dolores del Castillo [1] and Paloma Morales [2]

[1] Department of Food Analysis and Bioactivity, Institute of Food Science Research (CIAL, CSIC-UAM), 28049 Madrid, Spain.

[2] Department of Nutrition, Food Science and Food Technology, Faculty of Veterinary, Universidad Complutense de Madrid, 28040 Madrid, Spain.

Food Research International, 2017, 100, 791–797.
https://doi.org/10.1016/j.foodres.2017.08.012

Abstract

The aim of the present study was to validate the food safety of CSE, by studying its effect on cytotoxicity (100 – 20,000 µg/ml) and genotoxicity (10, 100 and 1000 µg/ml) and also to investigate its preventive potential (1, 10 and 100 µg/ml) against B(a)P induced DNA damage. Prior to analyses, the antioxidant capacity and the microbiological quality of CSE were tested. DNA damage (strand breaks and oxidized purines/pyrimidines) was evaluated by the alkaline single-cell gel electrophoresis or comet assay. HepG2 cells were pre-treated with CSE (1, 10 and 100 µg/ml) for 24 h followed by the addition of 100 µM B(a)P in presence of CSE for other 24 h. Detection of oxidized purines and pyrimidines was carried out using Formamidopyrimidine DNA glycosylase or Endonuclease III enzymes, respectively. Chlorogenic acid (CGA), the major antioxidant present in coffee, was used as a control. Treatment with 100 µM B(a)P significantly increased ($p < 0.05$) levels of DNA strand breaks and oxidized purine and pyrimidine bases. Treatment of HepG2 cells with CSE did not induce either cytotoxicity or genotoxicity. CSE significantly inhibited ($p < 0.05$) genotoxicity induced by B(a)P and the observed effect may be associated to its antioxidant capacity. CGA alone at the concentration present in CSE was effective against B(a)P. Thus, CGA seems to be a contributor to the preventive effect of CSE against B(a)P induced DNA damage in HepG2 cells. In conclusion, CSE presents potential as a natural sustainable chemoprotective agent against the chemical carcinogen B(a)P.

Keywords: benzo (a) pyrene, chlorogenic acid, coffee silverskin, DNA bases oxidative damage, food ingredient, genotoxicity

1. Introduction

The validation process for a novel food or ingredient established by the European legislation (EFSA Journal 2016;14(11):4594) comprises chemical characterization (toxic and health promoters), proposal for applications, *in vitro* assays, *in vivo* toxicity, *in vivo* bioactivity and human trials (Figure 1). In 2016, EFSA published a guidance for preparing and presenting scientific data for novel food applications. This guidance indicates the requirements needed when submitting an application for the authorization of novel foods: physicochemical, biochemical properties and microbiological characterization as well as data on the compositional, nutritional, toxicological and allergenic properties of the novel food (EFSA Panel on Dietetic Products, 2016). Information on genotoxicity is a key component in risk assessment of novel foods or ingredients. The purpose of genotoxicity testing for risk evaluation of substances in food is to identify compounds that could cause heritable damage in humans, to predict potential genotoxic carcinogens in cases where carcinogenicity data are not available, and to contribute to understand the mechanism of action of chemical carcinogens (EFSA Scientific Committee, 2011).

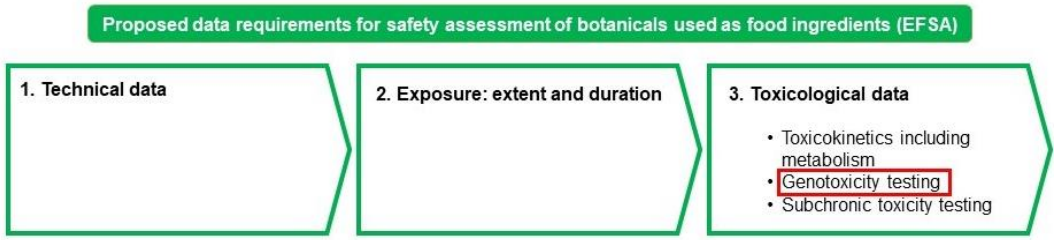


Figure 1. Scheme of the proposed data requirements for safety assessment of botanicals used as food ingredients (EFSA).

Coffee silverskin (CS) is a thin tegument of the outer layer of the two beans present in the coffee cherry. CS is the only by-product generated in the roasting process and it contains phytochemicals with antioxidant character such as chlorogenic acid (CGA). The extract (WO/2013/004873) prepared from Arabica coffee silverskin (CSE) is enriched in CGA and possesses high antioxidant power (del Castillo *et al*., 2016). The present study provides novel scientific information for supporting the usefulness of CSE as a sustainable natural source of bioactive compounds.

One of the mechanisms that leads to DNA lesions is oxidative stress associated with the activity of metabolic enzymes that can induce DNA strand breaks and oxidized bases (Xue & Warshawsky, 2005). Since CSE comes from a natural source and is known to

possess high antioxidant power, this extract could protect cells from DNA damage when induced by an oxidative agent.

HepG2 cells, a human hepatoblastoma cell line, are widely used in the field of xenobiotic metabolism for the study of cytotoxic or genotoxic agents (Cao *et al*., 2007). Benzo(a)pyrene (B(a)P) is the most widely used model compound for studying the effects of carcinogenic polycyclic aromatic hydrocarbons (PAHs) (Brinkmann *et al*., 2013). B(a)P is formed during incomplete combustion or pyrolysis of organic material and it is found in air, water, soils and in thermally processed foods and cigarette smoke (International Agency for Research on Cancer (IARC), 2012). B(a)P is a DNA-reactive chemical that interacts with DNA. Strand breaks or alkali labile sites, including abasic sites, may result from the action of reactive oxygen species (ROS) that arise during metabolism of food mutagens in cells (Haza & Morales, 2013).

The aim of the present study is to give new information related to CSE safety in order to use it as a food ingredient, as well as to provide scientific data to support a cause-effect relationship of the antioxidant power claim made on coffee and their derivatives. We also investigated the chemoprotective potential of CSE against B(a)P induced DNA damage in HepG2 cells and the contribution of CGA in CSE as a chemoprotective agent.

2. Materials and methods

2.1. Chemicals

Chlorogenic acid (CGA), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), benzo(a)pyrene (B(a)P), dimethyl sulfoxide (DMSO) and low melting point agarose (LMP) were purchased from Sigma–Aldrich (St Louis, MO, USA). Formamidopyrimidine-DNA glycosylase (Fpg) and Endonuclease III (Endo III) were obtained from Trevigen Inc. (Gaithersburg, MD, USA). Culture medium and supplements required for the growth of HepG2 cells were purchased from Lonza (Lonza Group, Basel, Switzerland). MTT Proliferation Kit I was purchased from Roche (Indianapolis, IN, USA).

2.2. Preparation of soluble coffee silverskin extract

Coffee silverskin from Arabica species was provided by Fortaleza S.A. (Spain). CSE was produced as described in the patent WO 2013/004873 (del Castillo *et al*., 2013). Briefly, 50 mg of coffee silverskin were added per H_2O milliliter. This mixture was stirred for 10 minutes at 100 ºC, filtered and the filtrate was freeze-dried. Powdered CSE was prepared in aqueous solution, sterile filtered and added to medium to achieve final

concentrations between 1 and 20,000 µg/ml. CSE chemical composition is described in Table S1 (Supplementary Material). CGA is the major antioxidant present in coffee; therefore, it was used as an antioxidant control.

2.3. Overall antioxidant capacity of coffee silverskin extract

The trapping capacity of cationic free radicals was evaluated using the method of radical $ABTS^{\bullet+}$ bleaching described by Re *et al*., (1999) and modified by (Oki *et al*., 2006) for its use in a microplate. Aqueous solutions of CGA (0.15 – 2.0 mmol/l) were used for calibration. Absorbance was measured in microplate using a UV-Visible Spectrophotometer (BioTek Instruments, Winooski, VT, USA). All measurements were performed in triplicate and results were expressed as % CGA eq. (w/w).

2.4. Microbiological analyses

CSE was microbiologically analyzed to evaluate the safety of its use as a food ingredient. Count of (1) total aerobic microorganisms, (2) aerobic microorganisms forming endospores and (3) molds and yeasts were carried out. All assays were performed in sterile conditions and with previous solubilization of 10 g of CSE in BPW (90 ml) by using a stomacher (230 rpm, 1 min). Different conditions were set for each analysis: (1) pour plate method, PCA medium, incubation at 30ºC 72 h; (2) pour plate, BHI agar medium, preincubation (80 ºC, 10 min) and incubation at 37ºC 48h; and (3) spread method, SDA with chloramphenicol and incubation at 25ºC 120 h. Results were expressed as colony forming units (CFU)/g.

2.5. Cell culture

Human hepatocellular carcinoma (HepG2) cells were purchased from the Biology Investigation Center Collection (CIB, Madrid, Spain). Only cells of passage 10-17 were used in the experiments. Cells were cultured as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % v/v heat inactivated fetal calf serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin and 1 % v/v L-glutamine. Cell cultures were incubated at 37 ºC and 100 % humidity in a 5 % CO_2 atmosphere.

2.6. Cytotoxicity

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Cell Proliferation Kit I, Roche, Indianapolis, IN, USA) to select non-toxic concentrations of CSE and CGA. First, HepG2 cells were cultured at a density of 1 x 10^5 cells per well of a 96-well plate for 24 h. Then, cells were treated with CSE (0 – 20,000 µg/ml) or CGA (0 – 10,000 µg/ml) for 24 h. Subsequently,

cells were incubated in MTT Labeling Reagent for 4 h at 37 ºC and then, 100 µl of solubilization solution were added. After 24 h, the optical density of each well was read at 620 nm (test wavelength) and 690 nm (reference wavelength) using a microplate reader. Experiments were carried out in triplicate (n = 16). Results were expressed as the percentage of viability (% SDH) with respect to the control (medium treated cells).

2.7. DNA damage

The comet assay was carried out according to the protocol of Olive *et al*. with minor modifications (Olive *et al*., 1992). Briefly, HepG2 cells were plated on 24 well plates at a density of 1.5 x 10^5 cells/ml culture medium. Twenty-four hours after seeding, cells were exposed to CSE (1 – 1,000 µg/ml), or CGA (1 – 1,000 µg/ml), for another 24 h at 37 ºC and 5 % CO_2. After incubation, 12 µl of a suspension of 1 x 10^5 cells were mixed with 70 µl of LMP agarose type VII (0.75 % in PBS), distributed on a LMP agarose type VII (0.3 % in PBS) pre-coated slide, and left to set on an ice tray. Three slides were prepared for each concentration of the compound tested, one slide for control and the others slides to be treated with Fpg or Endo III. After solidification, cells were lysed in darkness for 1 hour in a high salt alkaline buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X-100, pH 10). Slides were then equilibrated 3 x 5 minutes in enzyme buffer (0.04 M HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8). Then, slides were incubated with 30 µl of Fpg or Endo III at 1 µg/ml in enzyme buffer for 30 minutes at 37 ºC in a humid dark chamber. Control slides were incubated with 30 µl enzyme buffer. Following enzyme treatment, slides were placed in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13, cooled in a refrigerator) in darkness for 40 minutes. Electrophoresis was performed in a cold-storage room, in darkness, in a Bio-Rad subcell GT unit containing the same buffer, for 30 minutes at 25 V. After electrophoresis, slides were neutralized using 0.4 M Tris pH 7.5 and fixed in methanol. Subsequently, DNA was stained with ethidium bromide (10 µg/ml) in Tris acetate EDTA (TAE 1X) for 5 minutes and examined in a fluorescence microscope (OLYMPUS BH-2) connected to a computerized image analysis system (Comet Score 1.0). Percent (%) DNA in the tail was used as the parameter for DNA damage analysis using the software. The level of Fpg and Endo III sites was obtained by subtracting the value of % DNA in the tail obtained without enzymes from the value obtained when enzymes were present. B(a)P (100 µM) was used as positive control.

2.8. Analysis of DNA damage induced by simultaneous treatment with B(a)P and CSE or CGA in alkaline comet assay

HepG2 cells were seeded into 24-multiwell plates at a density of 1.5×10^5 cells/ml culture medium. After 24 hours, CSE or CGA (1– 100 µg/ml) were added and plates were incubated for 24 hours at 37 ºC and 5 % CO_2. After incubation, cells were treated simultaneously with CSE or CGA and B(a)P 100 µM for other 24 hours. After the treatments, cells were then processed as described above.

2.9. Statistical Analysis

Images of 50 randomly selected cells per concentration were evaluated and the test was carried out three times. Untreated cells (C_0) were considered as negative controls. The reported Tail DNA is the mean ± standard error (SE) of three independent experiments. One-way analysis of variance (ANOVA) was carried out and statistical comparisons of the different treatments were performed using Tukey's test. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using the R package software environment Version 3.2.0 (http://www.r-project.org/).

3. Results

3.1. CSE overall antioxidant capacity

The *in vitro* antioxidant capacity of the CSE was evaluated by the $ABTS^{\bullet+}$ radical cation decolorization assay in order to assess the reproducibility of CS extraction. An overall antioxidant capacity value of 138.73 ± 14.8 mg CGA equivalents/gram of CSE was obtained for the trapping capacity of cationic free radicals of CSE. This result confirms that the patented CSE possesses in vitro antioxidant properties due to the presence of antioxidant molecules such as phenolic compounds, mainly CGAs, and melanoidins (Table S1, Supplementary Material).

3.2. Microbiological analyses of CSE

Results showed values of 3.25×10^5 CFU/g for endospores, 4.3×10^3 CFU/g of total aerobic microorganisms and a content of yeasts and molds lower than 10^2 CFU/g.

3.3. Evaluation of CSE and CGA cytotoxicity

Figure 2 shows data on CSE cytotoxicity in HepG2 cells obtained by the MTT method. The studied concentrations of CSE were 100-20,000 µg/ml. Since CGA has been detected in CSE and the antioxidant capacity of this extract has been associated to this compound among others, its effect on the viability of HepG2 cells was also studied (1-

10,000 µg/ml). Concentrations of CSE up to 10,000 µg/ml did not affect the viability of HepG2 cells (>80 %), but a remarkable significant decrease ($p < 0.05$) was observed for CSE at 20,000 µg/ml. The inhibitory concentration required to reduce cell viability 50 % (IC_{50}) after 24 hours of extract incubation was 13,158 µg/ml (Figure 2A). On the other hand, CGA also resulted non-cytotoxic at concentrations up to 5,000 µg/ml and the IC_{50} was 8,050 µg/ml (Figure 2B). When CGA (1 - 1,000 µg/ml) was added to cells, a significant increase ($p \leq 0.05$) in cell viability was observed. Therefore, to evaluate the genotoxicity and the possible protective effect of CSE and CGA on DNA damage, concentrations between 1 and 1,000 µg/ml were chosen.

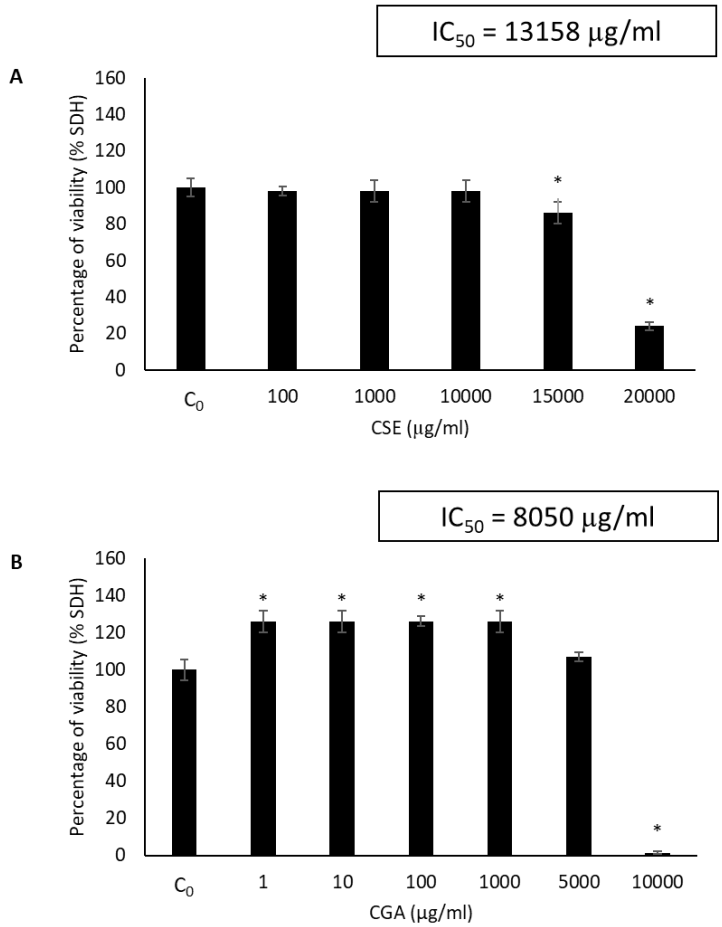


Figure 2. Effect of CSE (A) and CGA (B) on HepG2 cell viability by the MTT assay. Cells were cultured with different concentrations of CSE or CGA for 24 h. C_0 untreated cells. Data are shown as the mean ± SD of three independent experiments. Asterisks indicate a significant difference from the control (Tukey test, * $p \leq 0.05$).

3.4. Analysis of DNA damage induced by CSE or CGA in alkaline comet assay

Figure 3A shows the effect of CSE and CGA on the induction of strand breaks assessed by the comet assay without any enzyme treatment.

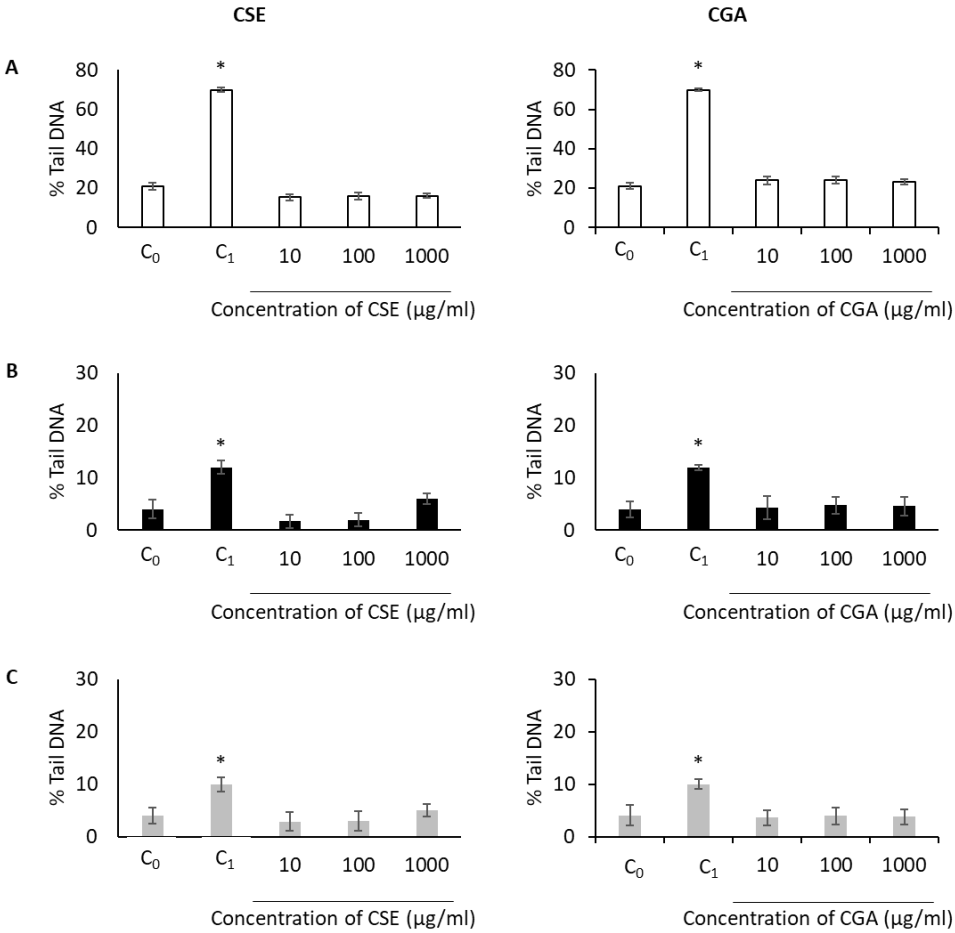


Figure 3. (A) Induction of DNA strand breaks by CSE or CGA on human HepG2 cells incubated without enzymes. C_0, untreated cells without enzymes. C_1, HepG2 cells treated with B(a)P 100µM and incubated without enzymes. (B) Oxidative DNA damage by CSE or CGA on human HepG2 cells incubated with Fpg enzymes. C_0, untreated cells with Fpg enzymes. C_1, HepG2 cells treated with BaP (100µM) and incubated with Fpg enzymes. (C) Oxidative DNA damage by CSE or CGA on human HepG2 cells incubated with Endo III enzymes. C_0, untreated cells with Endo III enzymes. C_1, HepG2 cells treated with BaP (100µM) and incubated with Endo III enzymes. The results are expressed as the mean ± SEM of three independent experiments for data points of % DNA in the tail of cells. Asterisks indicate significant difference from the control (Tukey test, $p \leq 0.05$).

A significant increase ($p \leq 0.05$) in strand breaks was observed when B(a)P 100 µM, used as positive control, was added to HepG2 cells (70 % tail DNA). None of the tested concentrations of CSE and CGA (10 - 1,000 µg/ml) resulted genotoxic to HepG2 cells (Figure 3A). There were no significant differences ($p > 0.05$) in strand breaks between untreated control cells and cells treated with CSE or CGA (10-1,000 µg/ml).

In order to determine if CSE or CGA produced oxidative DNA damage in purines or pyrimidines, DNA was digested with two repair specific enzymes Fpg (Figure 3B) or Endo III (Figure 3C) that recognize and cut oxidized DNA purines or pyrimidines, respectively. Cells treated with B(a)P 100 µM showed a significant increase ($p \leq 0.05$) in oxidized purines and pyrimidines (12 % and 10 % tail DNA, respectively). No significant oxidative DNA damage ($p > 0.05$) was produced in purines or pyrimidines when cells were treated with CSE or CGA. Therefore, none of the studied concentrations induced relevant strand breaks or oxidized purines or pyrimidines (Fpg or Endo III-sensitive sites, respectively) in the cells.

3.5. DNA damage induction by a combined treatment of B(a)P and CSE or CGA in the alkaline comet assay

The effect of CSE and CGA in B(a)P induced DNA damage in HepG2 cells is shown in Figure 4. Considering B(a)P induced DNA damage as 100 %, the maximum reduction of DNA strands breaks (26 % and 29 % for CSE and CGA, respectively) was observed at the highest concentration used (100 µg/ml). At all tested concentrations, DNA strand breaks were significantly reduced ($p \leq 0.05$) by the pretreatment with CSE and CGA showing a significant protective effect against B(a)P induced DNA damage.

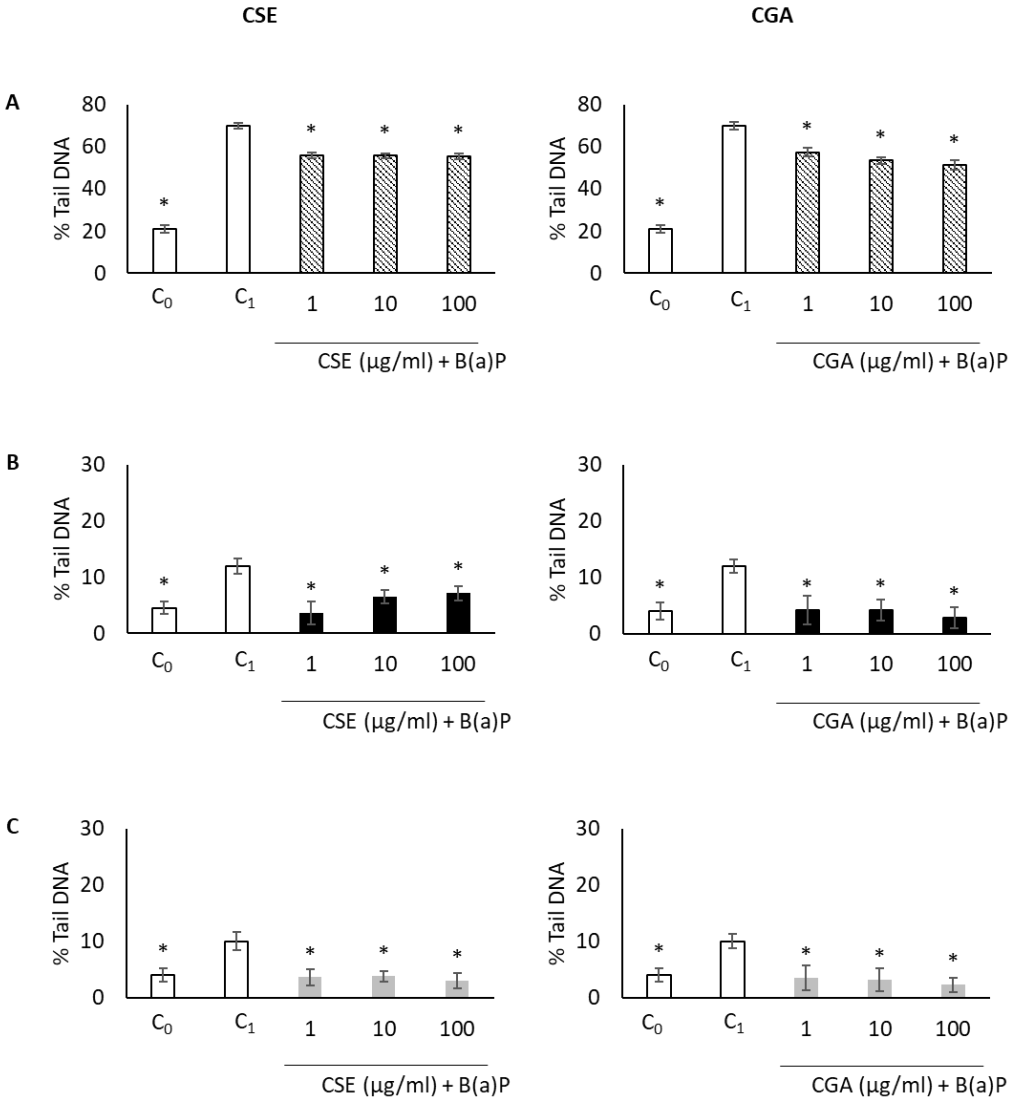


Figure 4. (A) Effect of CSE or CGA on BaP-induced DNA strand breaks in HepG2 cells. (C_0) Untreated cells without enzymes. (C_1) Cells treated with BaP (100 µM) and incubated without enzymes. (▧) Cells treated with BaP (100 µM) and CSE or CGA (1, 10 and 100 µg/ml) and incubated without enzymes. (B) Effect of CSE or CGA on BaP-induced oxidized purines in HepG2 cells. (C_0) Untreated cells with Fpg. (C_1) Cells treated with BaP (100 µM) and incubated with Fpg. (■) Cells treated with BaP (100 µM) and CSE or CGA (1, 10 and 100 µg/ml) and incubated with Fpg. (C) Effect of CSE or CGA on BaP-induced oxidized pyrimidines in HepG2 cells. (C_0) Untreated cells with Endo III. (C_1) Cells treated with BaP (100 µM) and incubated with Endo III. (▪) Cells treated with BaP (100 µM) and CSE or CGA (1, 10 and 100 µg/ml) and incubated with Endo III. Asterisks indicate significant difference from the control (C_1) (Tukey test, $p \leq 0.05$).

Both CSE and CGA in addition to decreasing DNA strand breaks, they significantly reduced *($p \leq 0.05$*) the formation of Fpg and Endo III sensitive sites. The maximum reduction of Fpg sensitive sites (69 % and 76 % for CSE and CGA, respectively, compared with the control) was observed at the lowest concentration of CSE (1 µg/ml) but at the highest concentration of CGA (100 µg/ml). However, Endo III sensitive sites were reduced (70 % and 77 % for CSE and CGA, respectively) in both cases by the highest doses of the tested compounds (100 µg/ml).

4. Discussion

In this work, we proved the microbiological quality and the non-cytotoxic and non-genotoxic character of CSE in order to give more information related to the safety of this extract as a novel food ingredient. CSE possesses high antioxidant power and data obtained in this study are in line with those previously described by other authors (Borrelli *et al*., 2004; del Castillo *et al*., 2016; Mesías *et al*., 2014).With regard to the biological contaminants that could be present in coffee, ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus ochraceus* and *Penicillium verrucosum* that tends to bioaccumulate along the food chain. OTA can induce renal toxicity, nephropathy, and immunosuppression, representing a risk for human safety. Therefore, its content in foods should be determined. Coffee is contaminated with OTA when coffee fruits fall onto the soil or during storage (Commission, 2006). Coffee is considered a secondary source of OTA in the human diet. However, OTA can be destroyed during roasting (Ferraz *et al*., 2010). Commission Regulation (EC) No 123/2005 defined OTA limits as 5 µg/kg for roasted coffee and 10 µg/kg for soluble coffee (Kyprianou, 2005). Since CSE is an extract from a coffee by-product, there is no specific OTA regulation limit; however, the absence of molds in the extract reduces the risk of OTA contamination. Therefore, CSE presented excellent microbiological quality becoming a safe food ingredient.

The study of the genotoxic potential is a basic component of chemical risk assessment (Louis Bresson *et al*., 2016). According to EFSA, the aim of genotoxicity testing of novel foods is to identify substances that could cause heritable damage in humans. The Scientific Committee recommends the Comet Assay as one of the suitable *ex vivo* tests for identifying substances that cause gene mutations and/or structural chromosomal aberrations. The *ex vivo* comet assay is easy to conduct and may be applied to any tissues from which single cell suspensions can be prepared. It is considered an indicator test to detect pre-mutagenic lesions and can be used for mechanistic studies (EFSA Scientific Committee, 2011). No previous research on genotoxicity of CS and its derivatives has been published.

The liver is the main organ of xenobiotic transformation. In consequence, liver cell lines are widely used in the field of xenobiotic metabolism such as genotoxicity studies (Mersch-Sunderman *et al*., 2004). HepG2 is a human hepatoblastoma cancer cell line that keeps many characteristics of hepatocytes such as the presence of enzymes that play key roles in the activation and detoxification of DNA-reactive carcinogens. Therefore, HepG2 cells represent a relevant *ex vivo* model to detect cytoprotective and non-genotoxic agents (Cao *et al*., 2007). Our results demonstrate that neither CSE nor CGA were cytotoxic for HepG2 cells up to concentrations of 10,000 and 1,000 µg/ml, respectively (Figure 2). These results are in agreement with studies using CSE in other human cell lines. For instance, concentrations of CSE and CGA up to 10 µg/ml and 10 µM respectively, did not affect the viability of pancreatic cells (INS-1E) (Fernandez-Gomez *et al*., 2016). Furthermore, studies using skin keratinocytes (HaCaT) have demonstrated that CSE is not cytotoxic when used at concentrations between 10 and 1,000 µg/ml (Iriondo-DeHond *et al*., 2016). Considering these results, in the present work cells were exposed to non-cytotoxic concentrations of CSE and CGA in the range of 1 to 1,000 µg/ml and DNA damage was evaluated. The results obtained in the comet assay indicated that both CSE and CGA did not cause a significant increase in DNA damage (strand breaks) after 24 h of treatment in HepG2 cells (Figure 3A). This is the first time the harmlessness and non-genotoxic effect of CSE is described. On the other hand, our results showed that CGA in concentrations present in CSE did not increase DNA strand breaks. Abraham *et al*., (2007) also confirmed the non-genotoxic profile of CGA in human promyelocytic leukemia cells (HL-60) when used at 200 µM.

Oxidatively damaged bases were detected by the comet assay by adding two repair specific enzymes, Fpg and Endo III, which are able to recognize the oxidized purines and pyrimidines, respectively. B(a)P (100 µM), positive oxidative control, induced a significant increase of DNA strand breaks, Fpg and Endo III sensitive sites in HepG2 cells. Burczynski and Penning (2000) showed that during the metabolic process, B(a)P produces ROS that can cause oxidative DNA damage and form adducts with DNA (Burczynski & Penning, 2000). Our results showed that CSE did not induce a significant increase of oxidation of purines and pyrimidines compared to the control (Figure 3B and 3C). However, a non-significant increase ($p > 0.05$) can be observed when cells were treated with CSE 1,000 µg/ml for 24 h. Moreover, CGA also did not increase oxidation of the purines and pyrimidines compared to the control in HepG2 cells.

In the present study, we evaluated the protective effect of CSE and CGA against B(a)P induced DNA damage (strand breaks and oxidized purines/pyrimidines) in HepG2 cells. Our results showed a significant decrease ($p \leq 0.05$) in DNA strand breaks when cells

were pretreated with CSE and CGA showing a significant protective effect against B(a)P induced DNA damage. Bakuradze *et al*., (2014) observed and confirmed the protective effect of roasted coffee consumption on DNA integrity in humans, as reported in a previous study. This study concluded that repeated coffee consumption is associated with reduced background DNA strand breakage (Bakuradze *et al*., 2011). The reduction of spontaneous DNA strand breaks observed in this study may be attributed to the presence of constituents with chemopreventive properties and antioxidants known to be present in coffee (such as chlorogenic acids and roast-associated constituents) (Bakuradze *et al*., 2014). Considering that CS keeps part of the polyphenolic compounds that are normal constituents of coffee beans, such as CGA (del Castillo *et al*., 2016), it is likely that this effect described for coffee brews is maintained also in CS.

CSE and CGA showed a higher protective effect against B(a)P induced oxidative DNA damage compared to that observed for the strand breaks. This could be due to the well-known antioxidant capacity of CSE (del Castillo *et al*., 2016) and CGA (European Food Safety Authority (EFSA), 2011). The mixture of compounds present in the CSE and CGA alone can reduce oxidative damage to DNA and therefore, decrease cellular oxidative stress. In CSE the main free phenolic compounds are CGA and caffeic acid, present in large amounts in green coffee beans. These phenolic compounds may react during roasting with polysaccharides and proteins forming melanoidins, which are rich in phenolic groups able to exert antioxidant activity (Borrelli *et al*., 2004). Therefore, it is possible that the observed protective effect is attributed to the combination of antioxidant compounds present in CSE. CS also contains flavonoids that could contribute to the observed protective effect (Costa *et al*., 2014). Delgado *et al*., (2008) reported that certain flavonoids are active against oxidized pyrimidines induced by B(a)P, but not against oxidized purines.

One mechanism of cellular protection against carcinogens is the activation of phase II detoxifying and antioxidant enzymes (Talalay, 2000). Fernandez-Gomez *et al*., (2016) provided novel information regarding the antidiabetic mechanism of action of CSE and its components CGA and caffeine. CSE (≥ 1 μg/ml) and CGA (≥ 5 μM) increased the enzymatic activity of glutathione peroxidase (GPx). These studies concluded that physiological concentrations of pure CGA (10 μM) were able to protect pancreatic cells against oxidative stress while same concentrations of caffeine were ineffective. Further research should be conducted to identify which compound/s are responsible for the observed benefits since CGA concentrations in CSE were not effective against induced oxidative stress in pancreatic cells (Fernandez-Gomez *et al*., 2016).

The induction of antioxidant enzymes is mediated by the antioxidant or electrophile response element (ARE/EpRE), located in the promoter region of many of these phase II genes. The transcription factor NF-E2 p45 subunitrelated factor 2 (Nrf2) plays a key role in ARE-mediated gene expression (Rushmore *et al*., 1991). Volz *et al*., (2012) have investigated the effect of a coffee extract rich in CGA and N-methylpyridinium (NMP), on the Nrf2-activating properties. In HT29 cells the enriched coffee extract increased nuclear Nrf2 translocation and enhanced the transcription of ARE-dependent genes such as NAD(P)H:quinone oxidoreductase and glutathione-S-transferase (GST)A1. Pure CGA has been identified as a potent activator of the Nrf2/ARE pathway, activating gene expression of different phase II enzymes in the same cell line (Boettler *et al*., 2011). In addition, in a pilot human intervention study data indicated an association between Nrf2 transcription and a decrease in oxidative DNA damage in peripheral blood lymphocytes observed after 4 weeks of daily coffee consumption (enriched with CGA and NMP), supporting the hypothesis that coffee-mediated effects on gene expression are of chemoprotective relevance (Volz *et al*., 2012). Since the CSE presents high antioxidant properties due to a mixture of molecules exerting synergic effect including CGA, the observed chemoprotective effect might be associated to its effect on the antioxidant enzymatic defense of the cells.

In conclusion, the present study confirms that treatment of HepG2 cells with CSE did not induce either cytotoxicity or genotoxicity. Our results indicate that CSE protects human cells from DNA strand breaks and oxidative DNA damage effects of B(a)P, and that free CGA or linked to other chemical structures seem to be contributors to the observed chemoprotective effect of CSE. This extract presents potential as a natural and sustainable food ingredient.

Acknowledgements

The SUSCOFFEE Project (AGL2014-57239-R): Sustainable coffee production and consumption: Valorization of coffee waste into food ingredients funded this work. A. Iriondo-DeHond is a fellow of the FPI predoctoral program of MINECO (BES-2015-072191).

References

Abraham, S. K., Schupp, N., Schmid, U., & Stopper, H. (2007). Antigenotoxic effects of the phytoestrogen pelargonidin chloride and the polyphenol chlorogenic acid. *Molecular Nutrition and Food Research, 51*(7), 880–887. https://doi.org/10.1002/mnfr.200600214.

Bakuradze, T., Boehm, N., Janzowski, C., Lang, R., Hofmann, T., Stockis, J.-P. P., … Eisenbrand, G. (2011). Antioxidant-rich coffee reduces DNA damage, elevates glutathione status and contributes to weight control: Results from an intervention study.

Molecular Nutrition and Food Research, *55*(5), 793–797. https://doi.org/10.1002/mnfr.201100093.

Bakuradze, T., Lang, R., Hofmann, T., Eisenbrand, G., Schipp, D., Galan, J., & Richling, E. (2014). Consumption of a dark roast coffee decreases the level of spontaneous DNA strand breaks: a randomized controlled trial. *European Journal of Nutrition*, *54*(1), 149–156. https://doi.org/10.1007/s00394-014-0696-x.

Boettler, U., Sommerfeld, K., Volz, N., Pahlke, G., Teller, N., Somoza, V., … Marko, D. (2011). Coffee constituents as modulators of Nrf2 nuclear translocation and ARE (EpRE)-dependent gene expression. *Journal of Nutritional Biochemistry*, *22*(5), 426–440. https://doi.org/10.1016/j.jnutbio.2010.03.011.

Borrelli, R. C., Esposito, F., Napolitano, A., Ritieni, A., & Fogliano, V. (2004). Characterization of a new potential functional ingredient: coffee silverskin. *Journal of Agricultural and Food Chemistry*, *52*(5), 1338–1343. https://doi.org/10.1021/jf034974x.

Brinkmann, J., Stolpmann, K., Trappe, S., Otter, T., Genkinger, D., Bock, U., … Luch, A. (2013). Metabolically Competent Human Skin Models: Activation and Genotoxicity of Benzo[a]pyrene. *Toxicological Sciences*, *131*(2), 351–359. https://doi.org/10.1093/toxsci/kfs316.

Burczynski, M. E., & Penning, T. M. (2000). Genotoxic Polycyclic Aromatic Hydrocarbon ortho-Quinones Generated by Aldo-Keto Reductases Induce CYP1A1 via Nuclear Translocation of the Aryl Hydrocarbon Receptor. *Cancer Research*, *60*(4).

Cao, J., Jiang, L.-P., Liu, Y., Yang, G., Yao, X.-F., & Zhong, L.-F. (2007). Curcumin-induced genotoxicity and antigenotoxicity in HepG2 cells. *Toxicon*, *49*(8), 1219–1222. https://doi.org/10.1016/j.toxicon.2007.02.006.

Commission, C.-C. A. (2006). Code of practice for the prevention and reduction of ochratoxin contamination in coffee. *Cac/Rcp*, 1–14. https://doi.org/10.1016/j.ijpvp.2006.08.01.

Costa, A. S. G. G., Alves, R. C., Vinha, A. F., Barreira, S. V. P. P., Nunes, M. a., Cunha, L. M., & Oliveira, M. B. P. P. P. (2014). Optimization of antioxidants extraction from coffee silverskin, a roasting by-product, having in view a sustainable process. *Industrial Crops and Products*, *53*, 350–357. https://doi.org/10.1016/j.indcrop.2014.01.006.

del Castillo, M. D., Fernandez-Gomez, B., Martinez-Saez, N., Iriondo-DeHond, A., Martirosyan, D. M., & Mesa, M. D. (2016). Coffee silverskin extract for aging and chronic diseases. In D. M. Martirosyan (Ed.), *Functional Foods in Health and Disease*. CreateSpace Independent Publishing Platform.

del Castillo, M. D., Ibañez, M. E., Amigo, M., Herrero, M., Plaza del Moral, M., & Ullate, M. (2013). *Application of products of coffee silverskin in anti-ageing cosmetics and functional food*. WO 2013/004873.

Delgado, M. E., Haza, A. I., Arranz, N., Garc??a, A., & Morales, P. (2008). Dietary polyphenols protect against N-nitrosamines and benzo(a)pyrene- induced DNA damage (strand breaks and oxidized purines/pyrimidines) in HepG2 human hepatoma cells. *European Journal of Nutrition*, *47*(8), 479–490. https://doi.org/10.1007/s00394-008-0751-6.

EFSA Panel on Dietetic Products, N. and A. (2016). Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. *EFSA Journal*, *14*(September), 11. https://doi.org/10.2903/j.efsa.2016.NNNN.

EFSA Scientific Committee. (2011). Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *In Vivo*, *9*(9), 1–68. https://doi.org/10.2903/j.efsa.2011.2379.

European Food Safety Authority (EFSA). (2011). Scientific Opinion on the substantiation of health claims related to coffee, including chlorogenic acids from coffee, and protection of DNA, proteins and lipids from oxidative damage (ID 1099, 3152, 4301), maintenance of normal blood glucose concentration. *EFSA Journal*, *9*(4), 2057. https://doi.org/10.2903/j.efsa.2011.2057.

Fernandez-Gomez, B., Ramos, S., Goya, L., Mesa, M. D., del Castillo, M. D., & Martín, M. Á. (2016). Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells. *Food Research International.* https://doi.org/.doi.org/10.1016/j.foodres.2016.03.006.

Ferraz, M. B. M., Farah, A., Iamanaka, B. T., Perrone, D., Copetti, M. V., Marques, V. X., … Taniwaki, M. H. (2010). Kinetics of ochratoxin A destruction during coffee roasting. *Food Control*, *21*(6), 872–877. https://doi.org/10.1016/j.foodcont.2009.12.001.

Haza, A. I., & Morales, P. (2013). Spanish honeys protect against food mutagen-induced DNA damage. *Journal of the Science of Food and Agriculture*, *93*(12), 2995–3000. https://doi.org/10.1002/jsfa.6129.

International Agency for Research on Cancer (IARC). (2012). Chemical Agents and Related Occupations. *In A review of human carcinogens* (Vol. 100, pp. 111–144). Lyon.

Iriondo-DeHond, A., Martorell, P., Genovés, S., Ramón, D., Stamatakis, K., Fresno, M., … Del Castillo, M. D. (2016). Coffee silverskin extract protects against accelerated aging caused by oxidative agents. *Molecules*, *21*(6), 1–14. https://doi.org/10.3390/molecules21060721.

Kyprianou, M. (2005). COMMISSION REGULATION (EC) No 123/2005 of 26 January 2005 amending Regulation (EC) No 466/2001 as regards ochratoxin A. *Official Journal of the European Union*, *2005*(123), 2004–2006. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R0123&qid=1478028660364&from=ES.

Louis Bresson, J., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., Ildico Hirsch-Ernst, K., … Vinceti, M. (2016). SCIENTIFIC OPINION Draft guidance on the preparation and presentation of an application for authorisation of a Novel Food EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) Novel Food Guidance Panel members. *EFSA Journal*. https://doi.org/10.2903/j.efsa.2016.NNNN.

Mersch-Sundermann, V., Knasmüller, S., Wu, X. J., Darroudi, F., & Kassie, F. (2004). Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology*, *198*(1–3), 329–340. https://doi.org/10.1016/j.tox.2004.02.009.

Mesías, M., Navarro, M., Martínez-Saez, N., Ullate, M., del Castillo, M. D., & Morales, F. J. (2014). Antiglycative and carbonyl trapping properties of the water soluble fraction of coffee silverskin. *Food Research International*, *62*, 1120–1126. https://doi.org/10.1016/j.foodres.2014.05.058.

Oki, T., Nagai, S., Yoshinaga, M., Nishiba, Y., & Suda, I. (2006). Contribution of b-Carotene to Radical Scavenging Capacity Varies among Orange-fleshed Sweet Potato Cultivars. *Food Science and Technology Research*, *12*, 156–160.

Olive, P. L., Wlodek, D., Durand, R. E., & Banáth, J. P. (1992). Factors influencing DNA migration from individual cells subjected to gel electrophoresis. *Experimental Cell Research*, *198*(2), 259–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1370267.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Rushmore, T. H., Morton, M. R., & Pickett, C. B. (1991). The antioxidant responsive element: Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *Journal of Biological Chemistry*, *266*(18), 11632–11639.

Talalay, P. (2000). Chemoprotection against cancer by induction of Phase 2 enzymes. *BioFactors*, *12*(1–4), 5–11. https://doi.org/10.1002/biof.5520120102.

Volz, N., Boettler, U., Winkler, S., Teller, N., Schwarz, C., Bakuradze, T., … Marko, D. (2012). Effect of coffee combining green coffee bean constituents with typical roasting products on the Nrf2/ARE pathway *in vitro* and *in vivo*. *Journal of Agricultural and Food Chemistry, 60*(38), 9631–9641. https://doi.org/10.1021/jf302258u.

Xue, W., & Warshawsky, D. (2005). Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. *Toxicology and Applied Pharmacology, 206*(1), 73–93. https://doi.org/10.1016/j.taap.2004.11.006.

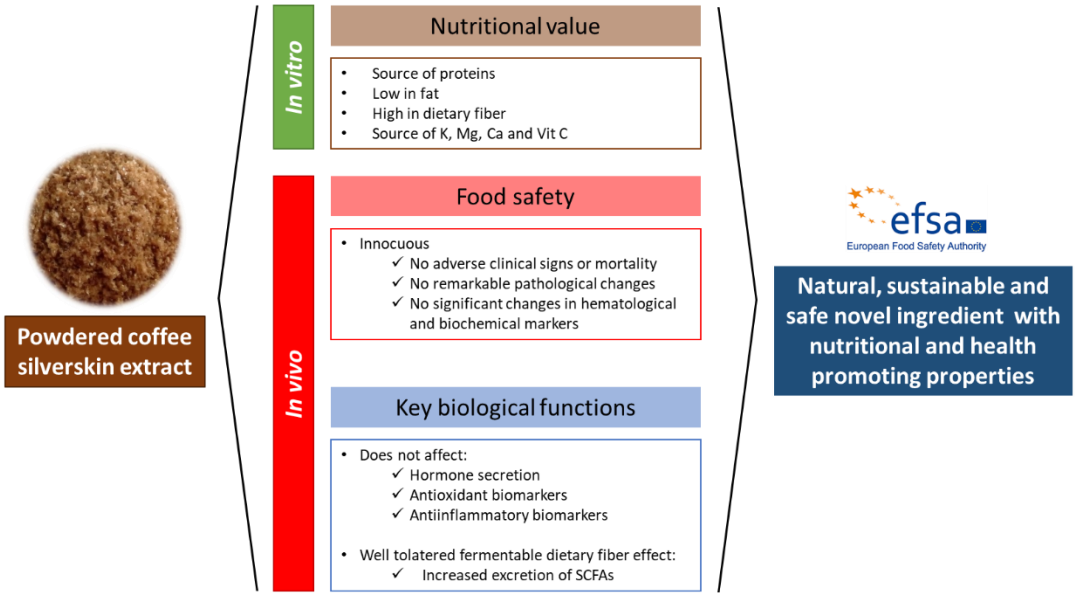
Table S1. Chemical composition of coffee silverskin extract (CSE).

Parameters	CSE (per 100g)
Proteins (g)	5.36
Carbohydrates (g)	5.44
Total dietary fibre (g)	28.69
Soluble dietary fibre (g)	24.01
Insoluble dietary fibre (g)	4.67
Caffeine (g)	3.02
Melanoidins (g)	17.26
Total CGAs* (g)	1.12
Total phenolic content (g)	3.10

*CGAs, chlorogenic acids.

Study 3: Coffee silverskin extract: Nutritional value, safety and effect on key biological functions

Iriondo-DeHond, A., Rios, M.B., Herrera, T., Rodriguez-Bertos, A., Nuñez, F., San Andres, M.I., Sanchez-Fortun, S., del Castillo, M. D. 2019. Coffee silverskin extract: Nutritional value, safety and effect on key biological functions. *Nutrients*, Submitted.



Coffee silverskin extract: Nutritional value, safety and effect on key biological functions

Amaia Iriondo-DeHond [1], Maria Belen Rios [1], Teresa Herrera [1], Antonio Rodriguez-Bertos [2,4], Fernando Nuñez [3], Manuel Ignacio San Andres [4], Sebastian Sanchez-Fortun [4], Maria Dolores del Castillo [1]

[1] Department of Food Analysis and Bioactivity, Institute of Food Science Research (CIAL, CSIC-UAM), 28049 Madrid, Spain.

[2] School of Veterinary Sciences. Health Surveillance Center (VISAVET). Complutense University. Puerta de Hierro Ave. 28040 Madrid, Spain.

[3] Centro de Biología Molecular Severo Ochoa (CBMSO, CSIC-UAM), 28049 Madrid, Spain.

[4] Facultad de Veterinaria, Universidad Complutense de Madrid, Av. Puerta de Hierro, s/n, 28040 Madrid, Spain.

Nutrients, 2019, Submitted.

Abstract

This research aimed to study the nutritional value, safety, effects on biochemical biomarkers and excretion of short chain fatty acids (SCFAs) *in vivo* of a coffee silverskin aqueous extract (CSE). A nutritional characterization of CSE (proteins, amino acids, fat, fatty acids, fiber, simple sugars and micronutrients) was carried out. Safety and biological effects were evaluated by a repeated dose study performed in Wistar rats. Hormone secretion (insulin, serotonin and melatonin), antioxidant (total antioxidant capacity, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase) and anti-inflammatory biomarkers (C-reactive protein) and the dietary fiber fermentability of CSE (analysis of SCFAs in feces) were analyzed in biological samples. CSE might be considered a source of proteins (16 %), potassium, magnesium, calcium and vitamin C, low in fat (0.44 %) and high in fiber (22 %). Its oral administration (1 g/kg) for 28 days was innocuous. Diet supplementation with CSE did not affect hormone secretion, antioxidant or anti-inflammatory biomarkers. Total SCFAs derived from CSE fiber fermentation were significantly higher ($p < 0.05$) in male rats compared to male control rats. CSE can be considered a natural, sustainable and safe food ingredient containing fermentable fiber able to produce SCFAs with beneficial effects on gut microbiota.

Keywords: antioxidant, coffee silverskin; food safety; novel food; nutrition; short-chain fatty acids

1. Introduction

According to the European Food Safety Authority (EFSA), 'Novel food' is defined as: "any food that was not used for human consumption to a significant degree within the Union before May 15th 1997 irrespective of the dates of accession of Member States to the Union" (EFSA Panel on Dietetic Products, 2016). In this sense, coffee silverskin (CS), a thin tegument of the outer layer of the coffee beans released during coffee roasting (Esquivel & Jiménez, 2012), may fall under the category of "food consisting of, isolated from or produced from plants or their parts". The application for authorizing the placing on the market within the European Union of a novel food and updating the Union list should include the name and description of the novel food, the description of the production process and the detailed composition of the novel food (EFSA Panel on Dietetic Products, 2016). Based on its nutritional and chemical composition, CS has the potential to be used as a food ingredient for human consumption within the concept of a healthy diet (Costa et al., 2018).

The major component in CS is dietary fiber (up to 55 %), which includes insoluble (≈45 %) and soluble (≈10 %) fiber (Ballesteros, Teixeira, & Mussatto, 2014; Jiménez-Zamora, Pastoriza, & Rufián-Henares, 2015). Dietary fiber is one of the main nutritional factors contributing to human well-being (Veronese et al., 2018). The EFSA defines dietary fiber as non-digestible carbohydrates, including non-starch polysaccharides, resistant starch and oligosaccharides and lignin (European Food Safety Authority (EFSA), 2010). According to the e-Library of Evidence for Nutrition Actions (eLENA) created by the World Health Organization (WHO), dietary fiber has important beneficial physiological effects that may contribute to the reduction of the risk of non-communicable chronic diseases (World Health Organization, n.d.). In the large intestine, dietary fiber is fermented by the microbiota leading to the generation of short-chain fatty acids (SCFAs) that also contribute to human well-being (Conlon & Bird, 2015). The high dietary fiber content of CS might benefit the intestine and gut microbiota (Ballesteros et al., 2014; Costa et al., 2018; Jiménez-Zamora et al., 2015). In this sense, previous studies have reported the prebiotic properties of this by-product, but the effect of CS intake on the excretion of SCFAs has not been previously described (Borrelli, Esposito, Napolitano, Ritieni, & Fogliano, 2004).

The second major component present in CS is protein (≈19 %), followed by carbohydrates (≈6 %) and fat (≈2 %) (Costa et al., 2018). CS is also a source of polyphenols, particularly chlorogenic acid (CGA) (588.9 mg/100 g) being 5-O-, 3-O- and 4-O-caffeoylquinic acids the most relevant (Mesías et al., 2014). Moreover, this by-product also contains caffeine (1 %) and melanoidins (5 %); the latter formed during the roasting process (Ateş & Elmacı, 2018b; Borrelli et al., 2004).

According to the European legislation, a 'health claim' means any claim that states, suggests or implies that a relationship exists between a food category, a food or one of

its constituents and health (The European Parliament and The Council of the European Union, 2006). However, the evaluation of the biological effects of foods and their impact on health is a limitation in nutrition studies. Nutritional biomarkers are used to objectively measure the nutritional status with respect to the intake or metabolism of dietary constituents in different biological samples (Picó, Serra, Rodríguez, Keijer, & Palou, 2019). These biomarkers also evaluate the bioavailability of a nutrient, which is determined by the biochemical analysis of its metabolites (Picó et al., 2019). The study of the metabolism of the bioactive compounds, caffeine and chlorogenic acid, present in an aqueous extract of CS (CSE) has been previously studied (Fernandez-Gomez, Lezama, et al., 2016). But to the best of our knowledge, the analysis of nutritional biomarkers after the intake of CSE has not been addressed yet.

All novel foods must be scientifically proven to be safe to public health and scientific evidence demonstrating that the novel food does not suppose a safety risk to human health should be included in the application for authorization (EFSA Panel on Dietetic Products, 2016). Data regarding the safety of the novel food such as toxicological studies, including repeated-dose toxicity, are required. The main objective of repeated-dose toxicity studies is to identify any adverse effects following prolonged exposure via an appropriate oral route (Parasuraman, 2011).

Therefore, the aim of the present research was to study the nutritional value of a potential novel ingredient, CSE, and to evaluate its safety and effects on key biological functions *in vivo*. To achieve this goal, a repeated dose study was carried out in Wistar rats and hormone secretion, antioxidant and anti-inflammatory biomarkers and the fiber effect of CSE were analyzed in biological samples. On the one hand, taking into account previous studies regarding the effect of CSE on carbohydrate metabolism (WO/2016/097450, 2016), the secretion of insulin after the repeated intake of this extract was measured. In addition, considering that CSE contains caffeine and that caffeine consumption leads to a decreased secretion of melatonin (Iriondo-DeHond, Aparicio García, et al., 2019; Shilo et al., 2002), levels of serotonin and melatonin were analyzed in blood samples of rats. On the other hand, since CSE is known for its antioxidant capacity and since oxidative stress and inflammation are closely related, antioxidant and anti-inflammatory biomarkers were also measured in rat's serum samples.

2. Materials and methods

2.1. Coffee silverskin extract (CSE)

Coffee silverskin from Arabica species was provided by Fortaleza S.A. (Spain). CSE was produced as described in the patent WO 2013/004873 (del Castillo et al., 2013). Briefly, 50 g of coffee silverskin were added per H_2O liter. This mixture was stirred for 10 minutes at 100 ºC, filtered and the filtrate was freeze-dried. The extract (yield = 14 % w/w of the initial sample) was stored at −20 °C until analysis.

2.2. Nutritional characterization of CSE

2.2.1. Protein content and amino acid composition

Content of proteins was determined by Kjeldahl mineralization followed by a colorimetric analysis of nitrogen for quantification (AOAC-32.1.22, 920.87). Results were expressed as % dry matter.

Amino acid analysis was performed according to AOAC-994.12 method, which is based on acid hydrolysis of sample followed by HPLC with post column derivatization using ninhydrin. Analysis in triplicate was performed and results were expressed as mg/g.

These analyses were performed according to that previously described (Martinez-Saez et al., 2017).

2.2.2. Fat and fatty acid profile

Total fat content was quantified by Soxhlet extraction with petroleum ether following the procedure described by the AOAC Official Method 945.16. Results are expressed as % dry matter.

Fatty acid profile was obtained by gas chromatography (Agilent 7820A GC System equipped with Flame Ionization Detector) analyses (Sukhija & Palmquist, 1988), calculated according to the ISO 12966-2:2017.

These analyses were performed according to that previously described (Iriondo-DeHond, Cornejo, et al., 2019).

2.2.3. Soluble simple sugars

Determination of sugars (mannose, glucose, xylose, fructose and sucrose) was carried out by ion exchange liquid chromatography (LC-IC). The LC system used in this study was Metrohm Advanced Compact ion chromatographic instrument (Metrohm, Switzerland) with a Pulsed Amperometric Detector (PAD) (Bioscan module, 817 IC. Metrohm), a pump (IC Pump 812), a coupled degasser (IC-837) and a Metrosep Carb 2 column (250 x 4 mm) packed with 5-µm spherical polymer beads. A mobile phase consisting of 100mM NaOH and 10 mM NaOAc was applied at a flow rate of 0.5 ml/min, and 20 µl of sample were injected in the LC-IC system. Data were analyzed using the Metrodata IC Net 2.3 software (Metrohm, Switzerland). This quantification was performed by the Analysis Service Unit facilities of the Institute of Food Science, Technology and Nutrition (ICTAN, CSIC, Madrid).

2.2.4. Dietary fiber

Insoluble (IDF), soluble (SDF) and total (TDF) dietary fiber were determined by using enzymatic-gravimetric assay based on the AOAC-991.43 and AACC-32.07.01 method. Results were expressed as %.

2.2.5. Ions and ascorbic acid

Determination of ions (sodium, potassium, magnesium and calcium) was carried out by ion exchange liquid chromatography (LC-IC) Metrohm Advanced Compact ion chromatographic instrument (867 IC. Metrohm, Switzerland) with a 819 Advanced IC Detector, a pump (IC Pump 818), a coupled degasser (IC-837) and a Metrosep C6 column (250 x 4 mm) packed with 5-µm spherical polymer beads (Metrohm AG, Switzerland). A mobile phase consisting of 3 mM metasulfonic acid was applied at 1 ml/min and 20 µl of sample were injected in the LC-IC system. Determination of ascorbic acid was performed using the same equipment and a Metrosep Organic Acids column (250 x 4 mm, 5-µm particle size) (Metrohm AG, Switzerland). The mobile phase was 50 mM lithium chloride at a flow rate of 0.5 ml/min. Data were analyzed using the Metrodata IC Net 2.3 software (Metrohm, Switzerland). This quantification was performed by the Analysis Service Unit facilities of the Institute of Food Science, Technology and Nutrition (ICTAN, CSIC, Madrid).

2.3. Repeated dose study

Healthy young adult male (n = 15) and female rats (n = 15) (3 weeks old) (Rattus norvegicus albinus), weighing 67.2 ± 4.7 g at the start of the experiment, were obtained from Charles River (Sant Cugat del Vallés, Spain). Animals were initially acclimated for seven days prior to the beginning of the experiment. Rats were housed in cages with a maximum capacity of five animals per cage under controlled temperature (22 ± 1 °C) in a 12-hour light/dark cycle with free access to standard food (A04 Safe Diets, Augy, France) and water ad libitum. Since it was not possible to replace the use of animals, all our effort was directed to minimize the number of animals used and any possible discomfort caused to them. The experimental protocols used were previously approved by the Ethics Committee on Animal Use (CEEA). The present study was approved by the Institutional Animal Ethics Committee (Reg. No. PROEX 011/17) of Community of Madrid, Spain.

The repeated dose toxicity study was carried out in accordance to OECD Test Guidelines 407 (Repeated Dose 28-day Oral Toxicity Study in Rodents) (Oecd/Ocde & Ction, 1995). The highest dose level was chosen to induce toxic signs. Thus, the dose level of powdered CSE in aqueous solution was 1 g/kg b.w. per day based on data from previous studies (Iriondo-DeHond, Aparicio García, et al., 2019). Rats were randomly allocated to two groups, control and treated with CSE, each consisting of 7 males and 7 females for the control group and 8 males and 8 females for the treated group. Animals received

sample (CSE 1 g/kg b.w.) or the vehicle (water) once daily by oral gavage for a total of 28 days. The dose volume was set at 1 ml/100 g b.w. Throughout the experiment, animals of both groups were observed daily before initiation of the administration period and once a week during the administration period, and detailed clinical signs were recorded. Body weight, food and water intake and behavior and signs of toxicity were recorded daily from the start to the 28th day of the experiment.

2.3.1. Safety

2.3.1.1. Necropsy, macroscopic analysis and organ weight

At the end of the treatment with CSE (1 g/kg b.w.) male and female rats were killed 1 day after the 28th administration. At the time of necropsy, animals were previously anesthetized with isoflurane (Forane®, 4 % induction dose using oxygen as a carrier gas and 2 % for maintenance during the process), and then total blood was collected by cardiac puncture. Rats were then sacrificed by exposure to excess carbon dioxide in a gas chamber. After euthanasia, a macroscopic assessment of the external body surface, cavities (thoracic, abdominal and cranial) and organs (change in position, shape, size, color and consistency) was carried out. Absolute and relative weight of the brain, lungs, liver, heart, spleen, thymus, kidneys, adrenal glands and sex organs was also determined. Relative organ weight was calculated using the following formula:

$$\textit{Relative organ weight (g \%)} = \textit{(weight of organ/weight of rat)} \times 100$$

2.3.1.2. Histopathological examination

Tissue samples from brain, lungs, liver, heart, spleen, thymus, kidneys, adrenal glands and sex organs were routinely processed for histology and fixed in buffered formalin 10% (Panreac©, Barcelona, Spain, stabilized with methanol at pH 7) for 24 hours at room temperature. Samples were then embedded in synthetic paraffin (Casa Alvarez, Madrid, Spain) with a melting point of 56 °C, using an automatic tissue processor (ASP300, Leica®, Wetzlar, Germany) with a program of automatic transmissions alcohols increased histological grading. Blocks were performed in a block forming unit (console Leica© EG1140H and cold plate Leica© EG1130) and 4 micron thick sections were obtained from rotation microtome Leica© brand, model RM 2155. Sections were deparaffinized in xylene and hydrated in alcohol and water. Conventional staining method, hematoxylin & eosin, was used by means of Leica© auto stainer SP4040. Then, dehydrated first ascending alcohol series and xylene were used, and finally, mounted with DPX (Nustain©, Nottingham, UK). The slides were then observed under light microscope.

2.3.1.3. Hematological and biochemical parameters

Blood samples were collected by cardiac puncture of anesthetized animals and stored in tubes with anticoagulant for determination of hematological parameters, and in tubes without anticoagulant to obtain serum for determination of biochemical parameters. Using laser flow cytometry technology (ADVIA 120 Hematology System, Siemens Healthineers, Getafe, Spain), the following parameters were examined: red blood cells (RBCs), hemoglobin concentration, hematocrit (HCT) value, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cells (WBCs), platelet count, mean platelet volume (MPV) and plateletcrit. Differential leukocyte count was determined by blood smear.

Measurements of plasma concentration, glucose, urea, creatinine, alanine-transferase (ALT), γ-glutamyl-transferase (GGT) and total cholesterol were performed by reflectance photometry (Reflotron®, Roche Diagnostics, Germany). Serum concentration of total proteins, albumin and bile acids was determined by liquid spectrophotometry (Biuret Test, bromocresol green technique and 3-α-hydroxysteroid dehydrogenase enzymatic technique, respectively), while the ionogram (sodium, potassium) was obtained by means of a blood gas analyzer (ABL90 Flex, Radiometer Medical ApS, Brønshøj, Denmark).

2.3.2. Effect on key biological functions

2.3.2.1. Hormone levels

Insulin levels were determined by a sandwich enzyme immunoassay kit (RayBio® Rat Insulin ELISA, Norcross, USA) according to the manufacturer's instructions. The kit was characterized by an analytical sensitivity of 5 μIU/ml and high analytical specificity (low-cross reactivity).

Serotonin levels were determined by a competitive enzyme immunoassay kit (General Serotonin Elisa Kit SAB Laboratories) according to the manufacturer's instructions. The kit was characterized by a detection range 1.56-100 ng/ml and no significant cross reactivity or interference was observed.

Melatonin levels were determined by a competitive enzyme immunoassay kit (Rat MT (Melatonin), Elabscience, Wuhan, China) according to the manufacturer's instructions. The kit was characterized by an analytical sensitivity of 9.38 pg/ml; detection range 15.63-1000 pg/mL and no significant cross reactivity or interference was observed.

2.3.2.2. Oxidative stress biomarkers

Non-enzymatic antioxidant capacity

The trapping capacity of cationic free radicals in serum samples was evaluated using the method of radical ABTS+ bleaching described by Re et al. (1999)(Re et al., 1999) and modified by Oki et al. (2006)(Oki, Nagai, Yoshinaga, Nishiba, & Suda, 2006) for its use in microplate. Aqueous solutions of Trolox (0.15–2.0 mM) were used for calibration. Absorbance was measured in microplate using a UV–Visible Spectrophotometer (BioTek Instruments, Winooski, VT, USA). All measurements were performed in triplicate, and results were expressed as eq. Trolox (mM).

Enzymatic oxidative stress biomarkers

Glutathione peroxidase (GPx) activity was measured in serum samples by the Glutathione Peroxidase Activity Assay Kit (MBL International, Woburn, USA) following the manufacturer's instructions. The assay had a detection sensitivity of ≈ 0.5 mU/ml of GPx in samples.

Glutathione reductase (GR) activity was measured in serum samples by the Glutathione Reductase Colorimetric Activity Assay Kit (MBL International, Woburn, USA) following the manufacturer's instructions. The assay could detect 0.1 – 40 mU/ml GR in samples.

Superoxide dismutase (SOD) activity was determined by SUPEROXIDE DISMUTASE Colorimetric Activity Kit (Arbor Assays, Ann Arbor, USA) in serum samples following the manufacturer's instructions. Sensitivity of the kit was 0.044 U/ml.

Catalase (CAT) activity was determined by CATALASE Colorimetric Activity Kit (Arbor Assays, Ann Arbor, USA) in serum samples following the manufacturer's instructions. Sensitivity of the kit was 0.052 U/ml and limit of detection was 0.062 U/ml.

2.3.2.3. Inflammation biomarkers

C - reactive protein (CRP) levels were determined by a sandwich enzyme immunoassay kit (Rat CRP Wuhan, China) according to the manufacturer's instructions. The kit was characterized by an analytical sensitivity of 0.19 ng/ml and high analytical specificity (low-cross reactivity).

2.3.2.4. Dietary fiber effect

Feces produced during 24 hours were collected from each cage the last day of the study. To evaluate the fiber effect of CSE, feces were counted and weighted. In addition, pH was measured since the level of fermentable carbohydrates and antioxidants in the diet are important factors affecting pH in feces. Antioxidant capacity of feces was determined by the ABTS+ method (section 2.3.2.2.).

Short chain fatty acids (SCFAs) were determined in 24 h feces from the treated and the control group collected the last day of the study. Five grams (wet weight) of rat feces were diluted in 50 ml of phosphoric acid 0.5 %, homogenized with an ultra-turrax (IKA®-Werke GmbH & Co. KG, Germany) for one minute, freezed with liquid nitrogen and after thawing, centrifuged at 4000 rpm for 20 minutes, and the supernatant was filtered through a membrane filter (pore size, 0.45 µm). SCFAs were identified and quantified by using gas chromatography (Agilent 7890A) coupled to mass spectrometry (Agilent 5975C) (Agilent Technologies, Santa Clara, CA, USA) with a DB-WAXtr column (60 m × 0.325 mm × 0.25 µm) (Agilent Technologies) (Primec, Mičetić-Turk, & Langerholc, 2017). The injector, ion source, quadrupole and the GC/MS interface temperature were 250, 230, 150 and 280 °C, respectively. The flow rate of helium carrier gas was kept at 1.5 ml/min. The initial column temperature was 50 °C and held 2 min, ramped to 150 °C at the rate of 15 °C/min, to 200 ºC at 5 ºC/min and then finally increased to 240 °C at the rate of 15 °C/min and kept at this temperature for 20 min. The ionization was carried out in the electron impact (EI) mode at 70 eV. The analytes were quantified in the selected ion monitoring (SIM) mode using the target ion and confirmed by confirmative ions. The target ion (m/z) of acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic and heptanoic acids are 43, 74, 43, 60, 60, 60, 60 and 60, respectively. Data were analyzed using MSD Chemstation E.02.00.493 program. The contents of SCFAs were calculated with standard methods.

2.4. Statistical analysis

Data were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was performed and statistical comparisons of the different treatments were performed using Tukey's test. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS Statistics 24.

3. Results

3.1. Nutritional characterization

With regard to the macronutrients present in CSE, Table 1 shows protein content, and free and total amino acids present in this by-product. CSE showed amounts of protein of 16.17 % (w/w). Considering free and total amino acids, 37 % and 34 % from the total corresponded to essential amino acids, respectively. Serine, asparagine and arginine presented the higher values of free amino acids. In addition, asparagine together with glutamic acid and glycine showed higher values for total amino acids. Free tryptophan levels found in CSE were 0.25 mg/g.

Table 1. Total protein (%) and free and total amino acid content (mg/g) of coffee silverskin extract (CSE).

	CSE	
Total protein (%)	16.17 ± 0.06	
Amino acids (mg/g)	**Free**	**Total**
Alanine (Ala)	0.66 ± 0.01	2.83 ± 0.13
Arginine (Arg)	0.79 ± 0.01	1.14 ± 0.06
Asparagine (Asp)	0.92 ± 0.02	6.41 ± 0.34
Cysteine (Cys)	0.18 ± 0.02	0.35 ± 0.01
γ-Amino butyric acid (GABA)	0.31 ± 0.00	N.D.
Glutamic acid (Glu)	0.77 ± 0.00	5.61 ± 0.31
Glycine (Gly)	0.23 ± 0.00	3.11 ± 0.14
Histidine (His)	0.19 ± 0.00	0.92 ± 0.04
Isoleucine (Ile)	0.21 ± 0.02	1.04 ± 0.06
Leucine (Leu)	0.26 ± 0.01	1.54 ± 0.09
Lysine (Lys)	0.59 ± 0.01	1.77 ± 0.10
Methionine (Met)	0.04 ± 0.03	0.57 ± 0.02
Phenylalanine (Phe)	0.29 ± 0.15	2.05 ± 0.09
Proline (Pro)	0.41 ± 0.02	2.32 ± 0.10
Serine (Ser)	1.32 ± 0.00	2.72 ± 0.14
Threonine (Thr)	0.19 ± 0.00	1.78 ± 0.09
Tryptophan (Trp)	0.25 ± 0.02	N.D.
Tyrosine (Tyr)	0.65 ± 0.22	1.48 ± 0.08
Valine (Val)	0.42 ± 0.01	1.79 ± 0.07
EAA (% total)	37.12 ± 0.46	33.70 ± 0.11
BCAA (Val + Leu + Ile) (% total)	10.18 ± 0.06	11.68 ± 0.05
AAA (Phe + Tyr + Trp) (% total)	13.60 ± 3.30	9.44 ± 0.39

EAA, essential amino acids; BCAA, branched-chain amino acids; AAA, aromatic amino acids. N.D., not determined. Results are expressed as mean ± SD (n = 2).

Fat content and the fatty acid profile of CSE is shown in Table S1. Values of fat content were of 0.44 %. In this study, C:16 was the main fatty acid found (26 %), followed by C18:2n6c (21 %), C22:0 (19 %), and C20:0 (12 %). In general, CSE mainly presents saturated fatty acids (SFA) (67 %), followed by polyunsaturated (PUFA) (26 %) and monounsaturated (MUFA) (7 %) fatty acids (Table S1).

Soluble simple carbohydrates detected in CSE were fructose (3.21 g/100 g), sucrose (1.97 g/100 g), glucose (1.29 g/100 g), mannose (0.06 g/100 g) and xylose (0.05 g/100 g).

TDF detected in CSE was 21.81 ± 0.77 %. Dietary fiber present in CSE is mainly composed of SDF (13.36 ± 20.3 %), representing the 61 % of TDF in CSE. Values of IDF in CSE were of 8.18 ± 0.17 %.

As for micronutrients in CSE, the presence of potassium (5,600 mg/100 g), magnesium (530 mg/100 g), sodium (460 mg/100 g) and calcium (350 mg/100 g) was also detected in this extract. In addition, results obtained from the analysis of organic acids showed values of 110 mg of ascorbic acid per 100 g of CSE.

3.2. Repeated dose study

3.2.1. Safety

No adverse clinical signs or mortality were observed in animals treated with CSE (1 g/kg b.w.) for 28 days.

3.2.1.1. Food and water intake

According to results shown in Table 2, animals orally treated with CSE (1 g/kg b.w.) did not show significant changes in food intake compared to the control group ($p > 0.05$). After 2 weeks, male rats presented significantly higher food intake ($p < 0.05$) than female rats. Taking into account the fiber content present in rat feed (3.9 %) and in CSE (22 %), male rats treated with CSE had a significantly increased intake of total dietary fiber ($p < 0.05$). However, this increased fiber consumption was not observed for female animals ($p > 0.05$). Considering water intake, no significant differences ($p > 0.05$) were observed during the first 3 weeks of the study. However, during the 4th week of treatment water intake of female rats treated with CSE was significantly higher ($p < 0.05$) than that observed for rats from the control group (Table 2).

Table 2. Food, fiber (3.9 % of fiber from diet) and water intakes of control rats (n = 15) and rats treated with CSE (1 g/kg b.w.) (n = 15) during repeated dose toxicity study.

	Male		**Female**	
	Control	**CSE**	**Control**	**CSE**
Food (g/rat/day)				
Week 1	14.1 ± 2.8 [a]	13.3 ± 1.4 [a]	15.5 ± 3.4 [a]	11.9 ± 0.9 [a]
Week 2	17.5 ± 3.0 [bc]	18.3 ± 1.2 [c]	15.2 ± 1.1 [ab]	14.2 ± 1.0 [a]
Week 3	21.3 ± 1.2 [b]	21.0 ± 2.7 [b]	17.7 ± 4.4 [ab]	16.2 ± 2.1 [a]
Week 4	22.3 ± 0.9 [b]	22.0 ± 1.0 [b]	16.5 ± 1.7 [a]	17.2 ± 1.7 [a]
Fiber intake (g/rat/day)				
From diet	0.9 ± 0.0 [a]	0.9 ± 0.0 [a]	0.7 ± 0.1 [a]	0.7 ± 0.1 [a]
From CSE	-	0.1 ± 0.0	-	0.1 ± 0.0
Total	0.9 ± 0.0 [a]	1.0 ± 0.0 [b]	0.7 ± 0.1 [a]	0.8 ± 0.1 [a]
Water (ml/rat/day)				
Week 1	21.6 ± 5.3 [a]	27.4 ± 9.2 [a]	24.1 ± 4.2 [a]	25.3 ± 9.0 [a]
Week 2	33.1 ± 9.8 [a]	30.1 ± 5.8 [a]	26.6 ± 8.6 [a]	31.9 ± 7.3 [a]
Week 3	43.0 ± 7.5 [a]	39.3 ± 9.9 [a]	32.9 ± 4.7 [a]	38.6 ± 5.7 [a]
Week 4	37.9 ± 6.3 [b]	41.0 ± 4.2 [b]	28.0 ± 4.2 [a]	35.5 ± 3.8 [b]

Data are expressed as the means ± standard deviation. Values in each row with different letters differ significantly (Tukey test, $p < 0.05$).

3.2.1.2. Body and organ weight

Figure 1 shows changes in absolute body weight of control and rats treated with CSE. At a dose of 1 g/kg b.w. of CSE, no significant changes were observed ($p > 0.05$) in treated rats compared to the control group up to the 28th day of the repeated dose toxicity study. After 2 weeks, treated and control male rats significantly increased ($p < 0.05$) their body weight compared to treated and control female rats. These results are in accordance to that observed in the food intake analysis (Table 2).

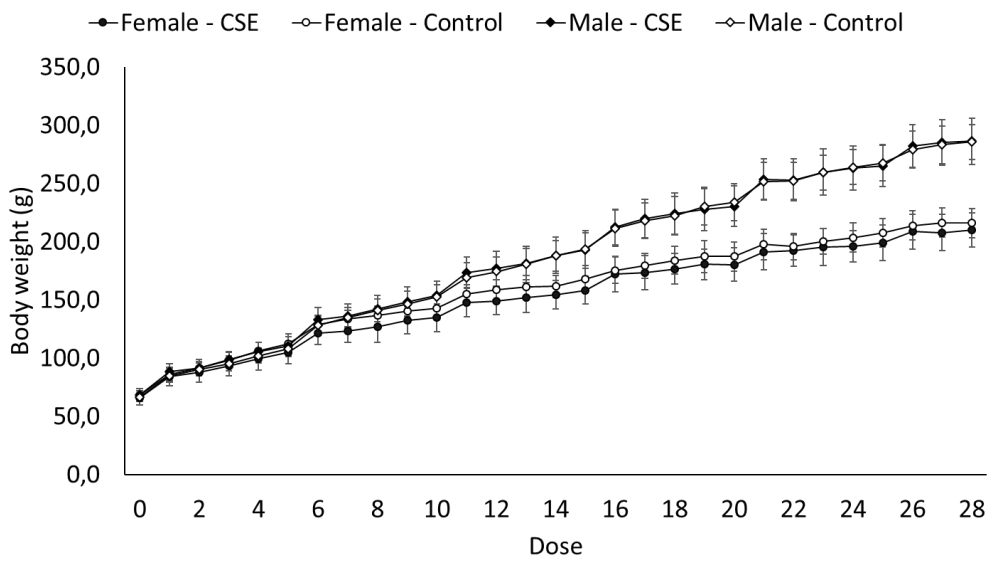


Figure 1. Body weight (g) of controls and rats treated with coffee silverskin extracts (CSE).

Mean and absolute organ weights from male and female rats of controls and rats treated with CSE (1 g/kg b.w.) are shown in Table 3. In both male and female rats, no significant change was observed in any organ weight ($p > 0.05$) in animals treated with CSE compared to the control group. In addition, post-mortem exams were performed in all of the animals to macroscopically observe any disturbances in vital organs (liver, heart, lungs, kidneys, thymus, adrenal glands, sex organs, brain and spleen) and to collect tissue samples for histology (liver, heart and kidneys). There were no gross findings that were considered to be related to the administration of CSE. Hemorrhage was found in lungs of control and treated rats, possibly derived from the sacrifice method employed.

Table 3. Body weights, absolute and relative organ weights (grams) of control rats (n = 15) and rats treated with CSE (1 g/kg) (n = 15).

	Male		**Female**	
Parameter	**Control**	**CSE**	**Control**	**CSE**
Body weight (g)	263.8 ± 15.0 [b]	261.7 ± 18.5 [b]	197.2 ± 10.4 [a]	191.4 ± 12.8 [a]
Absolute organ weights (g)				
Thymus	0.4 ± 0.1 [a]	0.6 ± 0.2 [a]	0.6 ± 0.2 [a]	0.5 ± 0.1 [a]
Lungs	1.7 ± 0.4 [ab]	2.1 ± 0.4 [b]	1.2 ± 0.6 [a]	1.5 ± 0.3 [ab]
Liver	10.0 ± 1.5 [b]	9.2 ± 1.3 [b]	6.6 ± 0.6 [a]	7.2 ± 1.0 [a]
Kidneys	1.0 ± 0.1 [ab]	1.4 ± 0.6 [b]	0.8 ± 0.1 [a]	0.8 ± 0.1 [a]
Adrenal glands	0.2 ± 0.0 [a]	0.2 ± 0.1 [a]	0.3 ± 0.1 [a]	0.2 ± 0.1 [a]
Sex organs	2.5 ± 0.2 [b]	2.7 ± 0.4 [b]	1.4 ± 0.3 [a]	1.1 ± 0.3 [a]
Brain	1.6 ± 0.1 [a]	1.6 ± 0.2 [a]	1.6 ± 0.2 [a]	1.5 ± 0.3 [a]
Heart	1.0 ± 0.1 [b]	0.9 ± 0.1 [ab]	0.8 ± 0.2 [ab]	0.7 ± 0.1 [a]
Spleen	0.5 ± 0.1 [a]	0.4 ± 0.1 [a]	0.5 ± 0.2 [a]	0.5 ± 0.1 [a]
Relative organ weights (g%)				
Thymus	0.2 ± 0.0 [a]	0.2 ± 0.1 [ab]	0.3 ± 0.1 [b]	0.2 ± 0.0 [ab]
Lungs	0.6 ± 0.2 [a]	0.8 ± 0.1 [a]	0.6 ± 0.3 [a]	0.8 ± 0.1 [a]
Liver	3.8 ± 0.4 [a]	3.5 ± 0.4 [a]	3.3 ± 0.3 [a]	3.7 ± 0.4 [a]
Kidneys	0.4 ± 0.0 [a]	0.5 ± 0.2 [a]	0.4 ± 0.0 [a]	0.4 ± 0.1 [a]
Adrenal glands	0.1 ± 0.0 [a]	0.1 ± 0.0 [a]	0.1 ± 0.1 [a]	0.1 ± 0.1 [a]
Sex organs	0.9 ± 0.1 [b]	1.0 ± 0.2 [b]	0.7 ± 0.2 [a]	0.6 ± 0.2 [a]
Brain	0.6 ± 0.0 [a]	0.6 ± 0.1 [a]	0.8 ± 0.1 [b]	0.8 ± 0.1 [b]
Heart	0.4 ± 0.0 [a]	0.3 ± 0.0 [a]	0.4 ± 0.1 [a]	0.4 ± 0.1 [a]
Spleen	0.2 ± 0.0 [a]	0.2 ± 0.0 [a]	0.3 ± 0.1 [a]	0.2 ± 0.1 [a]

Data are expressed as the means ± standard deviation. Values in each row with different letters differ significantly (Tukey test, $p < 0.05$).

3.2.1.3. Histopathology

At the end of the study, vital organs were subjected to histopathological examination. The microscopic observation showed no remarkable pathological changes in liver, kidneys or heart (Figure 2), or in the rest of the organs studied, from rats of both sexes and treated with CSE (1 g/kg b.w.) after repeated-dose toxicity study.

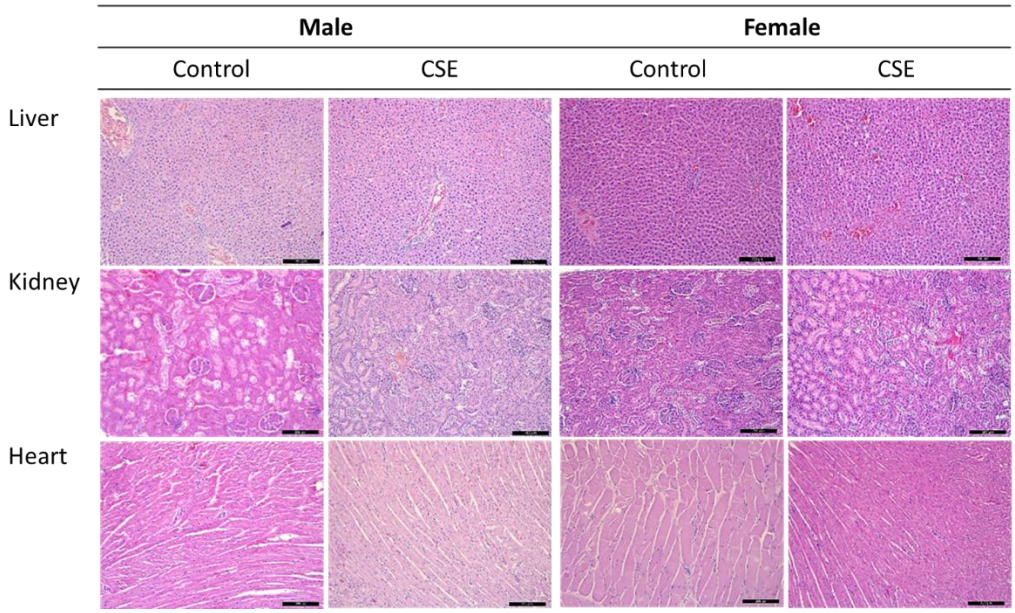


Figure 2. Representative microscopic findings for the liver, kidneys and heart of female and male Wistar rats treated with 1 g/kg b.w. CSE in aqueous solution and the controls for 28 days. Scale bar = 200μm.

3.2.1.4. Hematological and biochemical parameters

According to results described in Table 4, rats treated with CSE (1 g/kg b.w.) did not show statistically significant differences ($p > 0.05$) in the biochemical parameters studied in blood samples compared with control groups. Similar results were found in the hematological parameters also shown in Table 4, since no significant changes were observed in erythrocyte, leukocyte and platelet parameters ($p > 0.05$) of treated rats compared to the control group.

Table 4. Clinical biochemistry and hematology of blood samples from control rats (n = 15) and rats treated with CSE (1 g/kg) (n = 15).

	Male		Female	
	Control	**CSE**	**Control**	**CSE**
BIOCHEMICAL ANALYSES				
Glucose (mg/dl)	109.7 ± 42.2 [a]	101.0 ± 12.3 [a]	117.7 ± 7.9 [a]	106.0 ± 16.9 [a]
Urea (mg/dl)	41.2 ± 3.5 [ab]	38.5 ± 4.0 [a]	46.1 ± 6.4 [b]	39.2 ± 5.2 [ab]
Creatinine (mg/dl)	<0.5 [a]	<0.5 [a]	<0.5 [a]	<0.5 [a]
Proteins (g/dl)	6.0 ± 0.7 [a]	5.9 ± 0.4 [a]	6.1 ± 0.3 [a]	5.5 ± 0.2 [a]
ALT (U/l)	32.4 ± 6.6 [b]	26.6 ± 4.3 [ab]	23.1 ± 5.4 [a]	23.5 ± 3.5 [a]
GGT (U/l)	<5.0 [a]	<5.0 [a]	<5.0 [a]	<5.0 [a]
Cholesterol (mg/dl)	104.8 ± 4.5 [a]	103.3 ± 5.9 [a]	102.3 ± 3.6 [a]	105.6 ± 4.8 [a]
Potasium (mEq/l)	3.5 ± 0.2 [bc]	3.7 ± 0.5 [c]	2.8 ± 0.1 [a]	3.2 ± 0.3 [ab]
Sodium /mEq/l)	144.0 ± 4.6 [a]	145.2 ± 1.0 [a]	144.0 ± 1.1 [a]	142.2 ± 1.4 [a]
Albumin (g/dl)	3.4 ± 0.1 [ab]	3.3 ± 0.1 [a]	3.5 ± 0.2 [b]	3.2 ± 0.1 [a]
Bile acid (µmol/ml)	20.7 ± 10.9 [a]	15.0 ± 11.6 [a]	11.7 ± 4.5 [a]	14.0 ± 9.9 [a]
ERYTHROCYTE PARAMETERS				
RBCs (x10^6 /µl)	8.5 ± 0.5 [b]	8.1 ± 0.3 [ab]	7.9 ± 0.4 [ab]	7.8 ± 0.4 [a]
Hemoglobin (g/dl)	16.2 ± 0.4 [b]	16.1 ± 0.5 [b]	15.2 ± 0.6 [a]	14.8 ± 0.6 [a]
HCT (%)	48.4 ± 1.5 [b]	47.4 ± 1.2 [b]	44.5 ± 2.5 [a]	43.3 ± 2.3 [a]
MCV (fl)	56.6 ± 2.4 [ab]	58.3 ± 2.4 [b]	56.2 ± 0.9 [ab]	55.3 ± 1.4 [a]
MCH (pg)	19.0 ± 0.8 [a]	19.8 ± 0.8 [a]	19.2 ± 0.3 [a]	19.0 ± 0.8 [a]
MCHC (g/dl)	33.6 ± 0.3 [a]	34.1 ± 0.7 [a]	34.2 ± 0.6 [a]	34.3 ± 0.8 [a]
RDW (%)	11.8 ± 0.6 [b]	11.5 ± 0.4 [ab]	11.1 ± 0.3 [a]	11.6 ± 0.4 [ab]

	Male		Female	
	Control	**CSE**	**Control**	**CSE**
WBCs (x10^3 /µl)	8.1 ± 1.4 [b]	7.1 ± 3.0 [ab]	4.7 ± 1.4 [a]	4.6 ± 1.4 [a]
Segmented Neutrophils (µl)	1155.0 ± 303.5 [b]	586.3 ± 304.4 [a]	864.4 ± 396.2 [ab]	499.0 ± 226.2 [a]
Band Neutrophils (µl)	N.D.	N.D.	N.D.	N.D.
Lymphocytes (µl)	6919.0 ± 1323.6 [c]	6395.7 ± 2738.7 [bc]	3683.0 ± 1132.9 [a]	3996.8 ± 1550.2 [ab]
Monocytes (µl)	37.7 ± 47.5 [a]	71.0 ± 67.0 [a]	82.8 ± 82.3 [a]	57.1 ± 42.2 [a]
Eosinophils (µl)	59.7 ± 66.5 [a]	84.3 ± 98.7 [a]	69.2 ± 94.9 [a]	75.5 ± 18.4 [a]
Basophils (µl)	N.D.	N.D.	43.2 ± 93.9	N.D.
PLATELET PARAMETERS				
Platelets (x10^3 /µl)	801.8 ± 213.9 [a]	773.5 ± 309.3 [a]	763.0 ± 272.9 [a]	755.4 ± 368.4 [a]
MPV (fl)	9.0 ± 0.5 [a]	9.0 ± 0.8 [a]	8.7 ± 0.6 [a]	9.1 ± 0.4 [a]
Plateletcrit (%)	0.7 ± 0.1 [a]	0.6 ± 0.2 [a]	0.6 ± 0.2 [a]	0.6 ± 0.3 [a]

N.D. Non detected. Data are expressed as the means ± standard deviation. Values in each row with different letters differ significantly (Tukey test, $p < 0.05$). ALT, alanine aminotransferase; CSE, coffee silverskin extract; GGT, gamma-glutamyl transferase; HCT, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RBCs, red blood cells; RDW, red cell distribution width; WBCs, white blood cells.

3.2.2. Effect on key biological functions

3.2.2.1. Hormone levels

Evaluation of insulin, serotonin and melatonin levels after the 28 days repeated dose toxicity study did not report any differences in these hormone levels ($p > 0.05$) between treated and control rats (Table 5).

Table 5. Analyses of serum from control rats (n = 15) and rats treated with CSE (1 g/kg) (n = 15).

	Male		**Female**	
	Control	**CSE**	**Control**	**CSE**
Oxidative stress				
Total antioxidant capacity (Eq. Trolox mM)	2.0 ± 0.1 [a]	2.0 ± 0.1 [a]	2.0 ± 0.0 [a]	2.0 ± 0.1 [a]
Glutathione Peroxidase (GPx) (U/ml)	0.2 ± 0.0 [a]	0.2 ± 0.0 [a]	0.2 ± 0.0 [a]	0.2 ± 0.0 [a]
Glutathione Reductase (GR) (U/ml)	3.5 ± 0.8 [a]	3.9 ± 1.2 [a]	4.1 ± 0.9 [a]	3.7 ± 0.9 [a]
Superoxide Dismutase (SOD) (U/ml)	2.9 ± 0.8 [a]	3.1 ± 1.4 [a]	3.0 ± 1.3 [a]	3.2 ± 1.3 [a]
Catalase (CAT) (U/ml)	7.0 ± 4.2 [a]	7.4 ± 5.1 [a]	5.3 ± 1.5 [a]	7.1 ± 4.3 [a]
Inflammation				
C Reactive Protein (µl U/ml)	0.1 ± 0.0 [a]	0.1 ± 0.0 [a]	0.1 ± 0.0 [a]	0.1 ± 0.0 [a]
Hormones				
Insulin (ng/ml)	34.9 ± 19.3 [a]	29.4 ± 28.8 [a]	21.9 ± 12.6 [a]	26.9 ± 8.9 [a]
Serotonin (ng/ml)	240.8 ± 43.8 [a]	239.1 ± 46.0 [a]	208.4 ± 26.4 [a]	210.7 ± 20.2 [a]
Melatonin (pg/ml)	671.9 ± 258.1 [a]	853.4 ± 161.5 [a]	784.5 ± 196.3 [a]	900.6 ± 45.4 [a]

Data are expressed as the means ± standard deviation. Values in each row with different letters differ significantly (Tukey test, $p < 0.05$).

3.2.2.2. Oxidative stress and inflammation biomarkers

Table 5 shows the total antioxidant capacity (TAC) and the enzymatic oxidative stress biomarkers (glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT)) studied in serum samples of control and rats treated with CSE (1 g/kg b.w.). Daily administration of CSE (1 g/kg b.w.) did not significantly affect antioxidant biomarkers in blood samples ($p > 0.05$). In addition, no

significant differences ($p > 0.05$) between groups were observed in C reactive protein levels, suggesting no signs of inflammation when this particular biomarker was studied.

3.2.3. Dietary fiber effect

With regard to the analyses carried out in feces generated during 24 hours collected the last day of the study (Table 6), control male rats showed a significant higher ($p < 0.05$) number of pellets than male rats treated with CSE. No significant differences were detected in pellet number of female rats. In addition, no significant differences were detected in feces weight between groups, and treatment of male and female rats with CSE (1 g/kg b.w.) did not affect pH or total antioxidant capacity ($p > 0.05$) of rat feces collected at the end of the study (Table 6).

Table 6. Analyses of feces generated during 24 hours from control rats (n = 15) and rats treated with CSE (1 g/kg) (n = 15) collected the last day of the study.

	Male		**Female**	
	Control	**CSE**	**Control**	**CSE**
Number pellet/rat	53.0 ± 0.5 ^{b}	44.0 ± 1.6 ^{a}	44.0 ± 0.1 ^{a}	41.0 ± 2.0 ^{a}
Feces weight (g)/pellet	0.04 ± 0.0 ^{a}	0.03 ± 0.0 ^{a}	0.03 ± 0.0 ^{a}	0.03 ± 0.0 ^{a}
Feces weight (g)/rat	5.5 ± 0.2 ^{b}	5.2 ± 0.0 ^{ab}	4.2 ± 0.4 ^{a}	4.4 ± 0.2 ^{a}
pH	6.3 ± 0.1 ^{a}	6.3 ± 0.1 ^{a}	6.3 ± 0.1 ^{a}	6.3 ± 0.1 ^{a}
Total antioxidant capacity (Eq. Trolox mM)	8.2 ± 0.4 ^{a}	7.8 ± 0.3 ^{a}	8.1 ± 0.4 ^{a}	7.8 ± 0.7 ^{a}
SCFAs (µmol/g)				
Acetic	46.42 ± 1.75^{a}	68.49 ± 8.23^{c}	59.64 ± 1.79^{b}	62.18 ± 4.22^{bc}
Propionic	5.14 ± 0.24^{a}	5.94 ± 0.90^{a}	7.09 ± 0.34^{b}	5.29 ± 0.38^{a}
Isobutyric	0.24 ± 0.01^{a}	0.28 ± 0.04^{ab}	0.40 ± 0.02^{c}	0.31 ± 0.03^{b}
Butyric	6.71 ± 1.08^{c}	2.33 ± 0.20^{a}	10.63 ± 0.73^{d}	4.51 ± 0.34^{b}
Isovaleric	0.17 ± 0.02^{a}	0.16 ± 0.03^{a}	0.28 ± 0.01^{c}	0.21 ± 0.02^{b}
Valeric	0.49 ± 0.05^{b}	0.40 ± 0.06^{a}	0.78 ± 0.03^{c}	0.48 ± 0.04^{b}
Caproic	0.40 ± 0.04^{b}	0.30 ± 0.04^{a}	0.92 ± 0.03^{d}	0.55 ± 0.06^{c}
Heptanoic	N.D.	N.D.	N.D.	N.D.
Total SCFAs	59.57 ± 3.14 ^{a}	77.90 ± 9.42 ^{b}	79.75 ± 2.81 ^{b}	73.52 ± 5.05 ^{b}

Data are expressed as the means ± standard deviation. Values in each row with different letters differ significantly (Tukey test, $p < 0.05$).

Results of SCFAs analyzed in feces collected the last day of the study are shown in Table 6. The most abundant SCFAs found in feces were acetate, propionate and butyrate. Total

SCFAs (60 μmol/g and 78 μmol/g for control and treated rats) were significantly increased ($p < 0.05$) in feces when male rats were treated with CSE. However, this effect was not observed in female rats (79 μmol/g and 73 μmol/g for control and treated rats). In male rats, acetate significantly increased ($p < 0.05$) in feces after the repeated intake of CSE.

4. Discussion

4.1. Nutritional quality

Considering the nutritional profile of CS, this by-product has great potential for its use as a novel food ingredient (Borrelli et al., 2004; Martinez-Saez et al., 2014; Mussatto, Machado, Martins, & Teixeira, 2011; Pourfarzad, Mahdavian-Mehr, & Sedaghat, 2013). Results obtained in the present study suggest that the aqueous extract obtained from CS can be considered a 'source of proteins' since it contains over 12 g/100 g of proteins as stated in the Regulation (EU) No 1924/2006 (The European Parliament and The Council of the European Union, 2006) (Table 1). Protein values of CSE are similar to those of egg (12 %) and higher than protein content in milk (3 %) reported by the United States Department of Agriculture (USDA) National Nutrient Database. According to the Commission Regulation (EU) No 432/2012, the health claims for foods that are at least a source of proteins could be applied to CSE. These health claims are as follows: 'protein contributes to the growth and maintenance of muscle mass' and 'to the normal maintenance of normal bones' (The European Commission, 2012). The extract did not have a significant effect ($p > 0.05$) on protein intake biochemical parameters determined in blood samples from rats, urea and creatinine, compared to control rats (Table 4). Values obtained for these biomarkers were within the physiological range (Wolford et al., 1986).

To the best of our knowledge, this is the first study that shows the amino acid profile of CSE. The proportion of essential amino acids (34 – 37 %) makes CSE a good source of indispensable amino acids (Table 1). Claimed effects related to amino acids are growth or maintenance of muscle mass, maintenance of normal muscle function, faster recovery of muscle function/strength/glycogen stores after exercise, faster recovery from muscle fatigue after exercise and skeletal muscle tissue repair (Turck et al., 2018). However, these claimed effects are not established in terms of cause-effect relationship and have been evaluated by the EFSA Panel with an unfavourable opinion (Turck et al., 2018). CSE may be used as a novel ingredient in foods as a source of amino acids.

On the other hand, taking into account that the fat content of CS was 0.44 %, this extract could also be considered a 'low fat' product (The European Parliament and The Council of the European Union, 2006). Fat content in CSE was lower to that previously described for CS, which ranged from 1.6 % to 3.3 % (Costa et al., 2018; Napolitano, Fogliano, Tafuri, & Ritieni, 2007). Fatty acid composition of CS is mainly composed by palmitic

acid (C16:0), followed by linoleic acid (C18:2n6) and behenic acid (C:22:0), which agrees with that previously reported by other authors (Toschi, Cardenia, Bonaga, Mandrioli, & Rodriguez-estrada, 2014). As expected, the repeated intake of CSE (1 g/kg b.w.) did not have an effect on fat consumption biomarkers, such as cholesterol, aminotransferases and bile acids (Table 4), in rats treated with CSE compared to the control group.

The total amount of simple carbohydrates composing CSE (6.58 g/100g) did not rise ($p > 0.05$) glucose levels of treated rats compared to control rats (Table 4). The amount of total simple sugars is close to the value stated by the European Commission of 5 g of sugars per 100 g for reaching the nutrition claim of 'low sugar' (The European Parliament and The Council of the European Union, 2006). The value of simple sugars obtained in this study is similar to that obtained by Costa et al. (2018) (Costa et al., 2018). In contrast, this value is higher than that reported for a mixture of Arabica and Robusta CS by Toschi et al. (2014), although the main simple carbohydrates detected in both studies were fructose and sucrose (Toschi et al., 2014).

The amount of total dietary fiber obtained in this study (22 %); as well as SDF (14 %) and IDF (8 %), agrees with that previously reported for CSE (28 %, 24 % and 4 % for TDF, SDF and IDF, respectively) (Mesías et al., 2014). Different authors have reported the values of total, insoluble and soluble dietary fiber of CS, being approximately 60 %, 50 % and 8 %, respectively (Ballesteros et al., 2014; Borrelli et al., 2004; A. S. G. Costa et al., 2018; Martinez-Saez et al., 2014). Ballesteros et al. (2014) reported that insoluble dietary fiber in CS is composed by cellulose, hemicellulose and lignin (Ballesteros et al., 2014). This potential novel ingredient can reach the nutrition claim of 'high in fiber' in which the product has to contain at least 6 g of fiber per 100 g (The European Parliament and The Council of the European Union, 2006). The health claims attributed to the "high in fiber" nutrition claim are: 'fiber increases fecal bulk, contributes to normal bowel function and to an acceleration of the intestinal transit' (The European Commission, 2012).

The WHO recommends a potassium intake of at least 90 mmol/day (3.5 g/day) for adults to reduce of blood pressure and risk of cardiovascular disease, stroke and coronary heart disease in adults (World Health Organization, 2012). In this sense, CSE would be a good source of potassium (5.6 g/100 g) (European Council, 1990), which is in accordance to that reported by Costa et al. (2018) (Costa et al., 2018). According to the Commission Regulation (EU) No 432/2012, foods that are a source of potassium can be labeled under the following health claims: "potassium contributes to normal functioning of the nervous system, to normal muscle function and to normal blood pressure" (The European Commission, 2012).

On the other hand, the extract hereby studied may also be considered a 'source of magnesium, calcium and vitamin C'. Recommended daily allowances (RDAs) for

magnesium, calcium and vitamin C are 300 mg, 800 mg and 60 mg, respectively (European Council, 1990). Since values of magnesium, calcium and vitamin C present in CSE represent a 15 % of the recommended allowance per 100 g of product, this by-product may be considered a source of these compounds. The nutrition claim source of magnesium is related to the following health claims: "Magnesium contributes to a reduction of tiredness and fatigue, to electrolyte balance, to normal energy-yielding metabolism, to normal functioning of the nervous system, to normal muscle function, to normal protein synthesis, to normal physiological function, to the maintenance of normal bones and teeth and has a role in the process of cell division" (The European Commission, 2012).

Calcium is also involved in the health claims: "Calcium contributes to normal blood clotting, to normal energy-yielding metabolism, to normal muscle function, to normal neurotransmission, to normal function of digestive enzymes, has a role in the process of cell division and specialization and is needed for the maintenance of normal bones and teeth" (The European Commission, 2012).

Finally, health claims regarding vitamin C content are: "Vitamin C contributes to normal function of the immune system, to normal collagen formation for the normal function of blood vessels, bones, cartilages, skin and teeth; contributes to normal energy-yielding metabolism, to normal functioning of the nervous system, to normal physiological function, to the protection of cells from oxidative stress, to the reduction of tiredness and fatigue, to the regeneration of the reduced form of vitamin E and increases iron absorption" (The European Commission, 2012).

The strong correlation between diet and health, together with sedentary lifestyles, an aging population and increasing healthcare costs have driven the interest of research in developing healthier food products (Malla, Hobbs, & Kofi Sogah, 2018). Fiber-enriched foods have been developped in the past few years to increase dietary fiber consumption to reduce the risk of chronic diseases. CS has been employed as dietary fiber for reducing caloric density and increasing dietary fiber content of breads (Pourfarzad et al., 2013). CS has also been added to bread formulations as a natural sustainable source of antioxidants, α-glucosidase inhibitors and colorants (Guglielmetti, Fernandez-Gomez, Zeppa, & del Castillo, 2019). CS has also been used as a coloring and as a dietary fiber source, to achieve healthier nutritious and high sensorial quality biscuits. The nutritional value and the appearance of the biscuits also improved by the addition of CS (Garcia-Serna, Martinez-Saez, Mesias, Morales, & Castillo, 2014). CS has also been used in another bakery product, cakes have been formulated with up to 30 % of water treated CS as a flour substitute (Ateş & Elmacı, 2018b, 2018a). In addition, anti-obesity and antioxidant beverages have been developed with CSE. Beverages made from Arabica and Robusta CSE (100 µg/mL), reduced body fat by 21 % and 24 %, respectively, in Caenorhabditis elegans as an animal model (Martinez-Saez et al., 2014). Finally, recent

studies have used CS in yogurt production and showed that the bioactive compounds are still bioaccessible after the digestion process (Bertolino et al., 2019).

4.2. Safety

To the best of our knowledge, no study regarding the effects of the prolonged exposure of an aqueous extract of CS has been performed. In the present study, CSE from Arabica coffee beans was evaluated in Wistar rats exposed to 1 g/kg b.w. The feeding of CSE (1 g/kg b.w.) to male and female Wistar rats for 4 weeks did not cause mortality in any of the animals. This absence of mortality is considered a positive aspect to support a safe use of CSE in animals and in subsequent clinical trials with formulations containing this material.

The safety of these novel foods must be scientifically proven for human consumption. Previous studies have proven the safety CSE at different levels through genotoxicity and acute toxicity studies (Iriondo-DeHond, Aparicio García, et al., 2019; Iriondo-DeHond, Haza, Ávalos, del Castillo, & Morales, 2017). CSE did not induce either cytotoxicity or genotoxicity and also protected human cells from DNA strand breaks and oxidative DNA damage effects of chemicals agents such as benzo(a)pyrene (Iriondo-DeHond et al., 2017). In addition, no lethal effects were observed in acute toxicity studies when rats were treated with 2 g/kg b.w. by oral administration (Iriondo-DeHond, Aparicio García, et al., 2019).

With regard to food and water intake, there were no significant differences ($p > 0.05$) between treated and control groups, which indicates that CSE does not interfere with these parameters. Weight loss is considered of toxicological importance when the reduction is at least 10 % less than the initial body weight (Hayes & Kruger, 2014). As expected from the results obtained for food intake, body weight of rats treated with CSE did not change compared to the control group during the 28 days of the study ($p > 0.05$) suggesting that CSE does not compromise nutrient absorption. Other authors have reported the absence of changes in food intake and body weight between control rats and rats treated with β-glucans extracted from barley for 28 days (Delaney et al., 2003). El Kabbaoui et al. (2017) studied also the effect of a vegetal aqueous extract on Wistar rats and no significant changes in body weight were reported when rats were fed with 1 g/kg of *Cistus ladaniferus* L. extract (El Kabbaoui et al., 2017).

Significant changes in absolute and relative organ weight of rats can be considered as an important evidence of toxicity (Da Silva et al., 2016). Organs in this study were selected according to the Society of Toxicologic Pathology (STP) recommendations. With regard to the macroscopic morphological analysis, there was no change in the shape or weight of the studied organs (thymus, lungs, liver, kidneys, adrenal glands, sex organs, brain, heart and spleen) in rats treated with CSE (1 g/kg b.w.). Furthermore, the histopathology examination showed no histopathological lesions in liver, kidneys and heart after

treatment with CSE. The assessment of histopathological alterations in organs considered as a basic test in the safety assessment of tested materials (Al-Afifi, Alabsi, Bakri, & Ramanathan, 2018). These results reinforce the findings of absence of toxicity after treatment with 1 g/kg b.w. of this compound for 28 days. Other authors have also reported the absence of morphological or histopathological alterations when Wistar rats were treated with different plan extract following the same procedure described in this study (Afzan et al., 2012; El Kabbaoui et al., 2017; Khan et al., 2011).

Analysis of blood parameters in animal models has a high predictive value for alterations of the hematological system in human toxicity (Olson et al., 2000). No significant differences ($p > 0.05$) were observed in biochemical parameters when diet of rats was supplemented with CSE at 1 g/kg b.w. Glucose, cholesterol, proteins, potassium, sodium, albumin and bile acid levels in treated and control rats were in the same range of that previously described for Wistar rats (Boehm et al., 2007; Charles River Laboratories, 1998; Wolford et al., 1986). Kidney and liver functionality were evaluated by the measurement of urea and creatinine, and ALT and GGT, respectively. No significant changes ($p > 0.05$) were observed in these parameters when animals were treated with CSE 1 g/kg b.w. and the obtained values were similar to those in the reference databases (Boehm et al., 2007; Charles River Laboratories, 1998; Wolford et al., 1986). No signs of acute of prolonged hepatotoxicity were observed since liver enzyme levels were neither raised nor decreased (K. E. Imafidon and Okunrobo, 2012). These results are in accordance to the absence of toxicity observed in the histopathological analysis of kidney and liver tissue of rats treated with CSE 1 g/kg b.w. for 28 days. On the other hand, damage or destruction of blood cells negatively affects normal functioning of the body in both humans and animals (Al-Afifi et al., 2018). Analysis of erythrocytes, leukocytes and platelets showed the absence of alterations in these parameters after treatment with CSE.

4.3. Effect on key biological functions

With regard to hormone secretion, no significant differences ($p > 0.05$) were observed in insulin serum levels of healthy rats treated with CSE 1 g/kg for 28 days (Table 5). However, CSE has shown the ability to modulate insulin secretion in vitro in pancreatic INS-1E cells (Fernandez-Gomez, Ramos, et al., 2016). Doses of CSE of 1–10 μg/ml stimulated insulin secretion and reinforced the antioxidant defense in pancreatic beta cells stressed with streptozotocin (Fernandez-Gomez, Lezama, et al., 2016). In addition, previous studies have shown the anti-diabetic properties of CSE (Fernandez-Gomez, Lezama, et al., 2016; Fernandez-Gomez, Ramos, et al., 2016). Daily administration of CSE before the induction of diabetes with streptozotocin–nicotinamide (type 2 diabetes model) significantly reduced ($p < 0.05$) pancreatic oxidative stress protecting rats from developing diabetes (Fernandez-Gomez, Lezama, et al., 2016).

The essential amino acid tryptophan, which is present in CSE (Table 1), is the precursor of several important products including serotonin or melatonin (Paredes, Barriga, Reiter, & Rodríguez, 2009). Serotonin and melatonin are both hormones that regulate various biological functions such as sleep, appetite and mood. The melatonin-serotonin pathway affects appetite and digestive processes by endocrine as well as paracrine effects in both the brain and the gastrointestinal tract (Thor, Krolczyk, Gil, Zurowski, & Nowak, 2007). CSE used in this study possesses 3.4 mg/g dry matter of melatonin (del Castillo et al., 2016). However, no significant changes ($p > 0.05$) were observed in neither melatonin levels nor its precursor, serotonin, in serum of rats treated with CSE compared to the control group. CSE may be used as a source of tryptophan, which will act as a neurometabolite.

On the other hand, caffeine content in CSE is 24 mg/g (Iriondo-DeHond, Aparicio García, et al., 2019) and it has been reported that caffeine also impacts on circadian rhythms (Weibel et al., 2019). In humans, acute caffeine intake has been shown to delay the onset of melatonin secretion and decrease nighttime melatonin levels (Burke et al., 2015). Results obtained in this study seem to indicate that the physiological functions determined by these hormones were not affected by the repeated intake of CSE at 1 g/kg b.w.

Although CSE is known to possess high antioxidant capacity (Borrelli et al., 2004), non-enzymatic and enzymatic oxidative stress biomarkers (GPx, GR, SOD and CAT) analyzed in serum samples were not altered after the administration of CSE 1 g/kg for 28 days (Table 5). In contrast, previous studies have shown the antioxidant properties of CSE in vivo in pancreatic tissue samples of diabetic rats (Scalbert, Morand, Manach, & Rémésy, 2002). Further studies should be carried out to investigate the antioxidant properties of CSE in certain organs, since polyphenols are metabolized in tissues and these metabolites can also possess antioxidant properties (Scalbert & Williamson, 2000). In addition, no significant differences ($p > 0.05$) were observed in inflammation biomarkers, C reactive protein, in treated rats compared to the control group. Recent studies have reported that phenolic compounds composing CSE possess anti-inflammatory properties in vitro. CSE reduced the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and decreased the secretion of pro-inflammatory factors in LPS-stimulated RAW2643.7 macrophages (Rebollo-Hernanz, Zhang, Aguilera, Martín-Cabrejas, & Gonzalez de Mejia, 2019). Further research regarding the analysis of several pro-inflammatory biomarkers should be carried out in vivo.

Considering the potential fiber effect of CSE, no significant changes ($p > 0.05$) in feces number and weight were observed for female rats between groups after 28 days of CSE ingestion (Table 6). However, male rats treated with CSE showed a lower number of feces compared to the control group. Previous studies have shown that the high

molecular weight of CSE, rich in melanoidins, accelerated intestinal transit in treated animals after 28 days of exposure (Tores de la Cruz et al., 2019).

CS contains higher amount of soluble dietary fiber compared to other materials, and therefore, can be fermented and also possesses large water retention, promotes the growth of bifidobacteria, and decreases the absorption of fat and sugars (Ballesteros et al., 2014). Results regarding the analysis of SCFAs showed that sex seems to influence the SCFAs profile of rats' feces. Total SCFAs were significantly higher ($p < 0.05$) in female control rats compared to male control animals. Values of SCFAs and molar ratios of acetate:propionate:butyrate, which are also an indicator of dietary changes, of male rats showed in this study are similar to that previously described (Kleessen, Stoof, Schmiedl, Noack, & Blaut, 1994). Shastri et al. (2015) have previously reported the influence of sex in gut fermentation. Authors illustrated the need of considering sex in research studies that investigate health impacts from the intake of functional foods or ingredients that contribute to the improvement of gut health (Shastri, McCarville, Kalmokoff, Brooks, & Green-Johnson, 2015). Male rats treated with CSE showed significantly higher ($p < 0.05$) content of total SCFAs and acetate than male control rats. This is in agreement with the increased fiber consumption in male rats (Table 2). The higher levels of SCFAs had no significant effects ($p > 0.05$) on pH values obtained in feces of rats treated with CSE (Table 6). Health promoting properties associated to acetate are increased colonic blood flow and enhanced ileal motility (Scheppach, 1994). Considering the results obtained from the dietary fiber effect and the health claim made on foods high in fiber, it may be said that the dietary fiber present in CSE may contribute to normal bowel function.

With regard to butyrate, there is increasing evidence that this SCFA per se may be beneficial for human health (Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016). Lower values of butyrate ($p < 0.05$) were observed in animals treated with CSE. The majority of SCFAs are rapidly absorbed by the colonocytes in the cecum and large intestine and only approximately 5% are secreted in the feces, and the major part of butyrate is used as fuel for colonocytes and it plays an important role in maintaining colonic health in humans (den Besten et al., 2013; Jaquet, Rochat, Moulin, Cavin, & Bibiloni, 2009). Therefore, butyrate produced by fermentation of fiber present in CSE may have been absorbed by intestinal cells where it exerts is potential health benefits. Fiber from another coffee by-product, spent coffee grounds (SCGs), has shown anti-inflammatory properties after *in vitro* fermentation by human gut microflora. SCFAs produced by colonic fermentation of SCGs exhibited great anti- inflammatory properties by suppressing NO production, and inhibited inflammatory cytokines (López-Barrera, Vázquez-Sánchez, Loarca-Piña, & Campos-Vega, 2016). Considering results obtained in this study, CSE might be considered as a sustainable novel food ingredient with health promoting properties and with beneficial effects on gut microbiota.

5. Conclusions

The nutritional profile of CSE makes it a good candidate for its use as a novel food ingredient. This novel ingredient might be considered a source of proteins, low in fat and high in fiber. It can also be considered source of potassium, magnesium, calcium and vitamin C. Oral administration of CSE at a dose of 1 g/kg for a period of 28 days is safe in rats. Effects on key biological functions of CSE were studied in vivo in healthy rats, and the supplementation of the diet with CSE had no negative effects in hormone secretion, antioxidant or anti-inflammatory biomarkers. The dietary fiber effect of CSE has observed since total SCFAs and acetate derived from CSE fiber fermentation were significantly higher ($p < 0.05$) in male rats compared to male control rats. The same trend was observed in female rats for acetic acid. Altogether, coffee silverskin extract can be considered a natural, sustainable and safe food ingredient with potential effects for the gastrointestinal health due to the metabolites (SCFAs) derived from the fermentation of its dietary fiber. However, further clinical investigations are needed to confirm its safety and effectiveness in humans.

Acknowledgements

The SUSCOFFEE Project (AGL2014-57239-R): Sustainable coffee production and consumption: Valorization of coffee waste into food ingredients funded this work. A. Iriondo-DeHond is a fellow of the FPI predoctoral program of MINECO (BES-2015-072191). The authors thank Dr. Elena Hevia from the Animal facility of the Centro de Biología Molecular Severo Ochoa (CBMSO, CSIC). We are grateful to the Analysis Service Unit facilities of ICTAN and to the Servicio de Química de Proteínas of CIB for the analysis of Chromatography and Mass Spectrometry.

References

Afzan, A., Abdullah, N. R., Halim, S. Z., Rashid, B. A., Semail, R. H. R., Abdullah, N., … Ismail, Z. (2012). Repeated dose 28-days oral toxicity study of Carica papaya L. Leaf extract in Sprague Dawley rats. Molecules, 17(4), 4326–4342. https://doi.org/10.3390/molecules17044326

Al-Afifi, N. A., Alabsi, A. M., Bakri, M. M., & Ramanathan, A. (2018). Acute and sub-acute oral toxicity of Dracaena cinnabari resin methanol extract in rats. BMC Complementary and Alternative Medicine, 18(1), 1–14. https://doi.org/10.1186/s12906-018-2110-3

Ateş, G., & Elmacı, Y. (2018a). Coffee silverskin as fat replacer in cake formulations and its effect on physical, chemical and sensory attributes of cakes. Lwt, 90, 519–525. https://doi.org/10.1016/j.lwt.2018.01.003

Ateş, G., & Elmacı, Y. (2018b). Physical, chemical and sensory characteristics of fiber-enriched cakes prepared with coffee silverskin as wheat flour substitution. Journal of Food Measurement and Characterization, 0(0), 0. https://doi.org/doi.org/10.1007s11694-018-9988-9

Ballesteros, L. F., Teixeira, J. a., & Mussatto, S. I. (2014). Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. Food and Bioprocess Technology, 7(12), 3493–3503. https://doi.org/10.1007/s11947-014-1349-z

Bertolino, M., Barbosa-Pereira, L., Ghirardello, D., Botta, C., Rolle, L., Guglielmetti, A., … Zeppa, G. (2019). Coffee silverskin as nutraceutical ingredient in yogurt: its effect on functional properties and its bioaccessibility. Journal of the Science of Food and Agriculture, (February). https://doi.org/10.1002/jsfa.9659

Boehm, O., Zur, B., Koch, A., Tran, N., Freyenhagen, R., Hartmann, M., & Zacharowski, K. (2007). Clinical chemistry reference database for Wistar rats and C57/BL6 mice. Biological Chemistry, 388(5), 547–554. https://doi.org/10.1515/BC.2007.061

Borrelli, R. C., Esposito, F., Napolitano, A., Ritieni, A., & Fogliano, V. (2004). Characterization of a new potential functional ingredient: coffee silverskin. Journal of Agricultural and Food Chemistry, 52(5), 1338–1343. https://doi.org/10.1021/jf034974x

Burke, T. M., Markwald, R. R., McHill, A. W., Chinoy, E. D., Snider, J. A., Bessman, S. C., … Wright, K. P. (2015). Effects of caffeine on the human circadian clock in vivo and in vitro. Science Translational Medicine, 7(305), 305ra146-305ra146. https://doi.org/10.1126/scitranslmed.aac5125

Charles River Laboratories. (1998). Baseline Hematology and Clinical Chemistry Values for Charles River Wistar Rats - (CRL:(WI)BR) as a Function of Sex and Age. Wilmington.

Conlon, M. A., & Bird, A. R. (2015). The impact of diet and lifestyle on gut microbiota and human health. Nutrients, 7(1), 17–44. https://doi.org/10.3390/nu7010017

Costa, A. S. G., Alves, R. C., Vinha, A. F., Costa, E., Costa, C. S. G., Nunes, M. A., … Oliveira, M. B. P. P. (2018). Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product. Food Chemistry, 267, 28–35. https://doi.org/10.1016/j.foodchem.2017.03.106

Da Silva, A. P. D. S. C. L., Oliveira, G. L. D. S., Medeiros, S. C., Sousa, A. M. L., Lopes, L. D. S., David, J. M., … De Freitas, R. M. (2016). Pre-clinical toxicology of garcinielliptone FC, a tautomeric pair of polyprenylated benzophenone, isolated from Platonia insignis Mart seeds. Phytomedicine, 23(5), 477–482. https://doi.org/10.1016/j.phymed.2016.02.013

del Castillo, María Dolores, Fernandez-Gomez, B., Martinez-Saez, N., Iriondo-Dehond, A., Martirosyan, D. M., & Mesa, M. D. (2016). Coffee Silverskin Extract for Aging and Chronic Diseases. In D. M. Martirosyan (Ed.), Functional Foods for Chronic Diseases (pp. 386–409).

del Castillo, Maria Dolores, Fernandez-Gomez, B., Ullate, M., Mesa, M. D., & Del Castillo, M.D., Fernandez-Gomez, B., Ullate, M. and Mesa, M. D. (2016). WO/2016/097450. Oficina Española de Patentes y Marcas. Retrieved from https://patentscope.wipo.int/search/es/detail.jsf?docId=WO2016097450

del Castillo, María Dolores, Ibañez, M. E., Amigo, M., Herrero, M., Plaza del Moral, M., & Ullate, M. (2013). Application of products of coffee silverskin in anti-ageing cosmetics and functional food. WO 2013/004873.

Delaney, B., Carlson, T., Frazer, S., Zheng, T., Hess, R., Ostergren, K., … Jonker, D. (2003). Evaluation of the toxicity of concentrated barley b -glucan in a 28-day feeding study in Wistar rats, 41, 477–487.

den Besten, G., van Eunen, K., Groen, A. K., Reijngoud, D.-J., Venema, K., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of Lipid Research, 54(9), 2325–2340. https://doi.org/10.1194/jlr.r036012

EFSA Panel on Dietetic Products. (2016). Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. EFSA Journal Journal, 14(September), 11. https://doi.org/10.2903/j.efsa.2016.NNNN

El Kabbaoui, M., Chda, A., El-Akhal, J., Azdad, O., Mejrhit, N., Aarab, L., … Tazi, A. (2017). Acute and sub-chronic toxicity studies of the aqueous extract from leaves of Cistus ladaniferus L. in mice and rats. Journal of Ethnopharmacology, 209(April), 147–156. https://doi.org/10.1016/j.jep.2017.07.029

Esquivel, P., & Jiménez, V. M. (2012). Functional properties of coffee and coffee by-products. Food Research International, 46(2), 488–495. https://doi.org/10.1016/j.foodres.2011.05.028

European Council. (1990). Council Directive of 24 September 1990 on nutrition labelling for foodstuffs (90/496/ EEC). Official Journal of the European Union, (L), 18–63. Retrieved from http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32011R1169

European Food Safety Authority (EFSA). (2010). Scientific Opinion on Dietary Reference Values for carbohydrates and dietary fibre. EFSA Journal, 8(3), 1–77. https://doi.org/10.2903/j.efsa.2010.1462.Available

Fernandez-Gomez, B., Lezama, A., Amigo-Benavent, M., Ullate, M., Herrero, M., Martín, M. Á., … del Castillo, M. D. (2016). Insights on the health benefits of the bioactive compounds of coffee silverskin extract. Journal of Functional Foods, 25, 197–207. https://doi.org/10.1016/j.jff.2016.06.001

Fernandez-Gomez, B., Ramos, S., Goya, L., Mesa, M. D., del Castillo, M. D., & Martín, M. Á. (2016). Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells. Food Research International. https://doi.org/.doi.org/10.1016/j.foodres.2016.03.006

Garcia-Serna, E., Martinez-Saez, N., Mesias, M., Morales, F. J., & Castillo, M. D. Del. (2014). Use of Coffee Silverskin and Stevia to Improve the Formulation of Biscuits. Polish Journal of Food and Nutrition Sciences, 64(4), 243–251. https://doi.org/10.2478/pjfns-2013-0024

Guglielmetti, A., Fernandez-Gomez, B., Zeppa, G., & del Castillo, M. D. (2019). Nutritional quality, potential health promoting properties and sensory perception of an improved gluten free bread formulation containing inulin, rice protein and bioactive compounds extracted from coffee byproducts. Polish Journal of Food and Nutrition Sciences, Accepted. https://doi.org/10.31883/pjfns-2019-0012

Hayes, A. W., & Kruger, C. L. (2014). Hayes ' Principles Methods of Toxicology Hayes ' Principles Methods of Toxicology.

Iriondo-DeHond, A., Aparicio García, N., Velazquez Escobar, F., San Andres, M. I., Sanchez-Fortun, S., Blanch, G. P., … del Castillo, M. D. (2019). Validation of coffee by-products as novel food ingredients. Innovative Food Science and Emerging Technologies, 51, 194–204.

Iriondo-DeHond, A., Cornejo, F. S., Fernandez-Gomez, B., Vera, G., Guisantes-Batan, E., Alonso, S. G., … del Castillo, M. D. (2019). Bioaccesibility, Metabolism, and Excretion of Lipids Composing Spent Coffee Grounds. Nutrients, 11(6), 1411. https://doi.org/10.3390/nu11061411

Iriondo-DeHond, A., Haza, A. I., Ávalos, A., del Castillo, M. D., & Morales, P. (2017). Validation of coffee silverskin extract as a food ingredient by the analysis of cytotoxicity and genotoxicity. Food Research International, 100, 791–797. https://doi.org/10.1016/j.foodres.2017.08.012

Jaquet, M., Rochat, I., Moulin, J., Cavin, C., & Bibiloni, R. (2009). Impact of coffee consumption on the gut microbiota: A human volunteer study. International Journal of Food Microbiology, 130(2), 117–121. https://doi.org/10.1016/j.ijfoodmicro.2009.01.011

Jiménez-Zamora, A., Pastoriza, S., & Rufián-Henares, J. a. (2015). Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties. LWT - Food Science and Technology, 61(1), 12–18. https://doi.org/10.1016/j.lwt.2014.11.031

K. E. Imafidon and Okunrobo, L. O. (2012). Study on Biochemical Indices of Liver Function Tests of Albino Rats Supplemented with Three Sources of Vegetable Oils. Nigerian Journal of Basic and Applied Science, 19(2), 105–110. https://doi.org/10.1016/j.bbagen.2006.03.019

Khan, M. I., Denny Joseph, K. M., Muralidhara, Ramesh, H. P., Giridhar, P., & Ravishankar, G. A. (2011). Acute, subacute and subchronic safety assessment of betalains rich Rivina humilis L. berry juice in rats. Food and Chemical Toxicology, 49(12), 3154–3157. https://doi.org/10.1016/j.fct.2011.08.022

Kleessen, B., Stoof, G., Schmiedl, D., Noack, J., & Blaut, M. (1994). Feeding Resistant Starch Affects Fecal and Cecal Microflora and Short-Chain Fatty Acids in Rats 1 ABSTRACT : Blood, (April), 2453–2462.

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., & Bäckhed, F. (2016). From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. Cell, 165(6), 1332–1345. https://doi.org/10.1016/j.cell.2016.05.041

López-Barrera, D. M., Vázquez-Sánchez, K., Loarca-Piña, M. G. F., & Campos-Vega, R. (2016). Spent coffee grounds, an innovative source of colonic fermentable compounds, inhibit inflammatory mediators in vitro. Food Chemistry, 212, 282–290. https://doi.org/10.1016/j.foodchem.2016.05.175

Malla, S., Hobbs, J. E., & Kofi Sogah, E. (2018). Functional Foods, Health Benefits and Health Claims. Athens Journal of Health, 1(1), 37–46. https://doi.org/10.30958/ajh.1-1-3

Martinez-Saez, N., Tamargo García, A., Domínguez Pérez, I., Rebollo-Hernanz, M., Mesías, M., Morales, F. J., … del Castillo, M. D. (2017). Use of spent coffee grounds as food ingredient in bakery products. Food Chemistry, 216, 114–122. https://doi.org/10.1016/j.foodchem.2016.07.173

Martinez-Saez, N., Ullate, M., Martin-Cabrejas, M. a., Martorell, P., Genovés, S., Ramon, D., & Del Castillo, M. D. (2014). A novel antioxidant beverage for body weight control based on coffee silverskin. Food Chemistry, 150, 227–234. https://doi.org/10.1016/j.foodchem.2013.10.100

Mesías, M., Navarro, M., Martínez-Saez, N., Ullate, M., del Castillo, M. D., & Morales, F. J. (2014). Antiglycative and carbonyl trapping properties of the water soluble fraction of coffee silverskin. Food Research International, 62, 1120–1126. https://doi.org/10.1016/j.foodres.2014.05.058

Mussatto, S. I., Machado, E. M. S., Martins, S., & Teixeira, J. a. (2011). Production, Composition, and Application of Coffee and Its Industrial Residues. Food and Bioprocess Technology, 4(5), 661–672. https://doi.org/10.1007/s11947-011-0565-z

Napolitano, A., Fogliano, V., Tafuri, A., & Ritieni, A. (2007). Natural occurrence of ochratoxin A and antioxidant activities of green and roasted coffees and corresponding byproducts. Journal of Agricultural and Food Chemistry, 55(25), 10499–10504. https://doi.org/10.1021/jf071959+

Oecd/Ocde, & Ction, I. N. T. U. (1995). OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Guideline for Testing of Chemicals, 8(July), 1–8. https://doi.org/10.1787/9789264070684-en

Oki, T., Nagai, S., Yoshinaga, M., Nishiba, Y., & Suda, I. (2006). Contribution of b-Carotene to Radical Scavenging Capacity Varies among Orange-fleshed Sweet Potato Cultivars. Food Sci. Technol. Res, 12, 156–160.

Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., … Heller, A. (2000). Concordance of the toxicity of pharmaceuticals in humans and in animals. Regulatory Toxicology and Pharmacology, 32(1), 56–67. https://doi.org/10.1006/rtph.2000.1399

Parasuraman, S. (2011). Toxicological screening. Journal of Pharmacology & Pharmacotherapeutics, 2(2), 74–79. https://doi.org/10.4103/0976-500X.81895

Paredes, S. D., Barriga, C., Reiter, R. J., & Rodríguez, A. B. (2009). Assessment of the potential role of tryptophan as the precursor of serotonin and melatonin for the aged sleep-wake

cycle and immune function: Streptopelia risoria as a model. International Journal of Tryptophan Research, 2(1), 23–36.

Picó, C., Serra, F., Rodríguez, A. M., Keijer, J., & Palou, A. (2019). Biomarkers of nutrition and health: New tools for new approaches. Nutrients, 11(5), 1–30. https://doi.org/10.3390/nu11051092

Pourfarzad, A., Mahdavian-Mehr, H., & Sedaghat, N. (2013). Coffee silverskin as a source of dietary fiber in bread-making: Optimization of chemical treatment using response surface methodology. LWT - Food Science and Technology, 50(2), 599–606. https://doi.org/10.1016/j.lwt.2012.08.001

Primec, M., Mičetić-Turk, D., & Langerholc, T. (2017). Analysis of short-chain fatty acids in human feces: A scoping review. Analytical Biochemistry, 526, 9–21. https://doi.org/10.1016/j.ab.2017.03.007

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine, 26(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Rebollo-Hernanz, M., Zhang, Q., Aguilera, Y., Martín-Cabrejas, M. A., & Gonzalez de Mejia, E. (2019). Phenolic compounds from coffee by-products modulate adipogenesis-related inflammation, mitochondrial dysfunction, and insulin resistance in adipocytes, via insulin/PI3K/AKT signaling pathways. Food and Chemical Toxicology, 132, 110672. https://doi.org/10.1016/j.fct.2019.110672

Scalbert, A., Morand, C., Manach, C., & Rémésy, C. (2002). Absorption and metabolism of polyphenols in the gut and impact on health. Biomedicine & Pharmacotherapy, 56(6), 276–282. https://doi.org/10.1016/S0753-3322(02)00205-6

Scalbert, A., & Williamson, G. (2000). Dietary Intake and Bioavailability of Polyphenols. The Journal of Nutrition, 130(8), 2073S-2085S. https://doi.org/10.1093/jn/130.8.2073S

Scheppach, W. (1994). Effects of short chain fatty acids on gut morphology and function. Gut, 35(SUPPL. 1), 35–38. https://doi.org/10.1136/gut.35.1_suppl.s35

Shastri, P., McCarville, J., Kalmokoff, M., Brooks, S. P. J., & Green-Johnson, J. M. (2015). Sex differences in gut fermentation and immune parameters in rats fed an oligofructose-supplemented diet. Biology of Sex Differences, 6(1), 1–12. https://doi.org/10.1186/s13293-015-0031-0

Shilo, L., Sabbah, H., Hadari, R., Kovatz, S., Weinberg, U., Dolev, S., … Shenkman, L. (2002). The effects of coffee consumption on sleep and melatonin secretion. Sleep Medicine, 3(3), 271–273. https://doi.org/10.1016/S1389-9457(02)00015-1

Sukhija, P. S., & Palmquist, D. L. (1988). Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. Journal of Agricultural and Food Chemistry, 36(6), 1202–1206. https://doi.org/10.1021/jf00084a019

The European Commission. (2012). Commission Regulation (EU) No 432/2012. Official Journal of the European Union, 13(1924). Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32012R0432&from=EN

The European Parliament and The Council of the European Union. (2006). REGULATION (EC) No 1924/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on nutrition and health claims made on foods. Official Journal of the European Union, (404), 9–25. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1924&from=en

Thor, P. J., Krolczyk, G., Gil, K., Zurowski, D., & Nowak, L. (2007). Melatonin and serotonin effects on gastrointestinal motility. Journal of Physiology and Pharmacology, 58(SUPPL. 6), 97–103.

Tores de la Cruz, S., Iriondo-DeHond, A., Herrera, T., Lopez-Tofiño, Y., Galvez-Robleño, C., Prodanov, M., … Castillo, M. D. del. (2019). An Assessment of the Bioactivity of Coffee Silverskin Melanoidins. Foods, 8(2), 68. https://doi.org/10.3390/foods8020068

Toschi, T. G., Cardenia, V., Bonaga, G., Mandrioli, M., & Rodriguez-estrada, M. T. (2014). Coffee Silverskin : Characterization , Possible Uses , and Safety Aspects. Journal of Agricultural and Food Chemistry, 62, 10836–10844.

Turck, D., Castenmiller, J., De Henauw, S., Hirsch-Ernst, K. I., Kearney, J., Knutsen, H. K., … Siani, A. (2018). Guidance on the scientific requirements for health claims related to muscle function and physical performance: (Revision 1). EFSA Journal, 16(10). https://doi.org/10.2903/j.efsa.2018.5434

Veronese, N., Solmi, M., Caruso, M. G., Giannelli, G., Osella, A. R., Evangelou, E., … Tzoulaki, I. (2018). Dietary fiber and health outcomes: An umbrella review of systematic reviews and meta-analyses. American Journal of Clinical Nutrition, 107(3), 436–444. https://doi.org/10.1093/ajcn/nqx082

Weibel, J., Lin, Y.-S., Landolt, H.-P., Garbazza, C., Kolodyazhniy, V., Kistler, J., … Reichert, C. (2019). Caffeine-dependent changes of sleep-wake regulation: evidence for adaptation after repeated intake. BioRxiv, 641480. https://doi.org/10.1101/641480

Wolford, S. T., Schroer, R. A., Gohs, F. X., Gallo, P. P., Brodeck, M., Falk, H. B., & Ruhren, R. (1986). Reference range data base for serum chemistry and hematology values in laboratory animals. Journal of Toxicology and Environmental Health, 18(2), 161–188. https://doi.org/10.1080/15287398609530859

World Health Organization. (n.d.). e-Library of Evidence for Nutrition Actions (eLENA). Retrieved from https://www.who.int/elena/nutrient/en/

World Health Organization. (2012). Guideline: Potassium intake for adults and children. World Health Organization, (ISBN 978 92 4 150482 9), 52.

Table S1. Fat (%) and fatty acid content (g/100 g of FA methyl esters) of coffee silverskin extract (CSE).

	CSE
Fat (%)	0.44 ± 0.14
Fatty acid profile (g/100 g)	
C14:0	1.24 ± 0.03
C15:0	0.52 ± 0.00
C16:0	26.24 ± 0.02
C16:1n7	0.26 ± 0.01
C17:0	0.16 ± 0.01
C18:0	6.54 ± 0.02
C18:1n7c	0.84 ± 0.01
C18:1n9c	5.44 ± 0.10
C18:2n6c	20.89 ± 0.11
C18:3n3	0.96 ± 0.04
C20:0	12.86 ± 0.04
C20:1n9	0.37 ± 0.00
C20:5n3	0.18 ± 0.01
C21:0 / C20:3n6*	0.32 ± 0.00
C22:0	18.74 ± 0.04
C22:6n3	0.29 ± 0.07
C23:0	0.50 ± 0.01
C24:0 /C22:5n3*	3.63 ± 0.03
SFA	66.81 ± 0.01
MUFA	6.92 ± 0.10
PUFA	25.95 ± 0.11

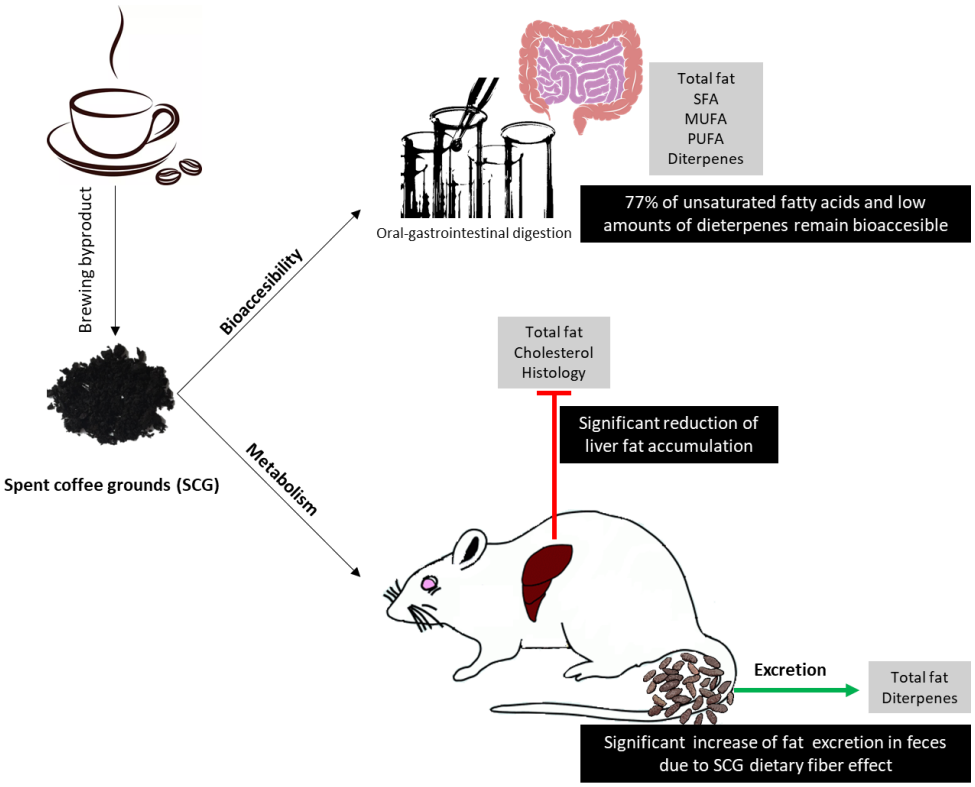
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Results are expressed as mean ± SD (n = 3).

CHAPTER 3

Study 4: Bioaccessibility, metabolism, and excretion of lipids composing spent coffee grounds

This chapter provides information regarding the validation of the coffee brewing by-product (spent coffee grounds, SCGs) as a safe, healthy and sustainable dietary fiber. Results from this chapter have been published in:

Iriondo-DeHond, A., Cornejo, F. S., Fernandez-Gomez, B., Vera, G., Guisantes-Batan, E., Alonso, S. G., San Andres, M.I., Sanchez-Fortun, S., Lopez-Gomez, L., Uranga, J.A., Abalo, R., del Castillo, M. D. (2019). *Nutrients*, 11(6), 1411. Available at: https://doi.org/10.3390/nu11061411



Bioaccessibility, metabolism, and excretion of lipids composing spent coffee grounds

Amaia Iriondo-DeHond [1], Fresia Santillan Cornejo [1], Beatriz Fernandez-Gomez [1], Gema Vera [2], Eduardo Guisantes-Batan [3], Sergio Gomez Alonso [3], Manuel Ignacio San Andres [4], Sebastian Sanchez-Fortun [4], Laura Lopez-Gomez [2], Jose Antonio Uranga [2], Raquel Abalo [2] and Maria Dolores del Castillo [1]

[1] Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM), Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain.

[2] Departamento de Ciencias Básicas de la Salud, Facultad de Ciencias de la Salud, Universidad Rey Juan Carlos (URJC), Alcorcón, 28922 Madrid, Spain.

[3] Instituto Regional de Investigación Científica Aplicada (IRICA), Universidad de Castilla-La Mancha, 13005 Ciudad Real, Spain.

[4] Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain.

Nutrients, 2019, 11(6), 1411. https://doi.org/10.3390/nu11061411

Abstract

The bioaccessibility, metabolism, and excretion of lipids composing spent coffee grounds (SCGs) were investigated. An analysis of mycotoxins and an acute toxicity study in rats were performed for safety evaluation. Total fat, fatty acids, and diterpenes (cafestol and kahweol) were determined in SCGs and their digests obtained *in vitro*. A pilot repeated intake study was carried out in Wistar rats using a dose of 1 g SCGs/kg b.w. for 28 days. Fat metabolism was evaluated by analysis of total fat, cholesterol, and histology in liver. The dietary fiber effect of SCGs was measured radiographically. The absence of mycotoxins and toxicity was reported in SCGs. A total of 77 % of unsaturated fatty acids and low amounts of kahweol (7.09 µg/g) and cafestol (414.39 µg/g) were bioaccessible after in vitro digestion. A significantly lower ($p < 0.1$) accumulation of lipids in the liver and a higher excretion of these in feces was found in rats treated with SCGs for 28 days. No lipid droplets or liver damage were observed by histology. SCGs acutely accelerated intestinal motility in rats. SCGs might be considered a sustainable, safe, and healthy food ingredient with potential for preventing hepatic steatosis due to their effect as dietary fiber with a high fat-holding capacity.

Keywords: bioaccesibility; cafestol; fatty acids; kahweol; lipid excretion; lipid liver biomarkers; lipid metabolism; spent coffee grounds

1. Introduction

Coffee is one of the major plantation crops grown worldwide and one of the most popular beverages consumed all over the world. When coffee is extracted in water for the preparation of the beverage, most of the hydrophobic compounds, such as oils, lipids, triglycerides and fatty acids and also insoluble carbohydrates like cellulose and other indigestible sugars, remain in the grounds (Padmapriya *et al.*, 2013).

Spent coffee grounds (SCGs) are the most abundant coffee by-product (45 %) generated during the treatment of coffee powder with hot water to prepare coffee infusion or steam for the instant coffee preparation (Murthy & Naidu, 2012). The main ingredient present in SCGs is dietary fiber and it can be categorized as antioxidant dietary fiber, useful as a potential dietary supplement (Campos-Vega *et al.,* 2015; del Castillo *et al.* 2014; Martinez-Saez *et al*., 2017). SCGs dietary fiber is mainly composed of insoluble fiber (88 % total dietary fiber), representing the 41.6 % of the total sample. SCGs are rich in mannose, galactose, glucose and arabinose polymerized into hemicellulose and cellulose (45.3 – 51.5 %, w/w) (Ballesteros *et al*., 2014; Mussatto *et al*., 2011) and there is also high content of galactomannans (Simões *et al*., 2013). In addition, lignin is also present in a significant amount in SCGs (19 – 26 % w/w) (Ballesteros *et al*., 2014; Pujol *et al*., 2013).

The second most predominant ingredient in SCGs are lipids, which are ranged from 10 to 30 % (Campos-Vega *et al.,* 2015; Martinez-Saez *et al*., 2017). The composition of lipids from SCGs slightly differs from that of raw coffee beans, but generally coffee oil contains mainly triacylglycerols (75 %) and unsaponificables, including about 19 % of total free and esterified diterpene alcohols, about 5 % of total free and esterified sterols, and very low amounts of other substances such as tocopherols (Calligaris *et al.,* 2009). Diterpenes, cafestol and kahweol, present in the lipid fraction of coffee are related to increased blood cholesterol, especially in the case of cafestol (George *et al*., 2008; Ricketts *et al.,* 2007). However, the diterpenes kahweol and cafestol are also known for their potential beneficial physiological effects such as ultraviolet B (UVB) skin protection, anticarcinogenic, anti-inflammatory and antioxidant activities (Silva *et al*., 2014).

The application of SCGs represents a value-added opportunity for coffee byproduct utilization at a very low cost. Several applications have been proposed for SCGs, which include their use as biofuels, biosorbents, animal feed, ceramic manufacturing and composite materials, among others (del Castillo *et al*., 2019; Kovalcik *et al*., 2018). Nevertheless, these approaches may actually be an inefficient way to use this biomass

waste. Considering the chemical composition of SCGs and its related potential health-promoting properties, it might be interesting to maintain the use of SCGs within the food industry sector. Therefore, SCGs have been proposed for their use as dietary fiber in bakery products such as bread, cookies and breakfast cereals, among others, giving rise to healthy and sustainable novel products (Martinez-Saez *et al*., 2017; Vázquez-Sánchez *et al*., 2018). The second main component of this coffee byproduct is fat and its bioaccesibility and in vivo effect might be modulated by the dietary fiber composing SCGs. Consequently, the aim of the present study was to determine the in vitro bioaccessibility of lipids and diterpenes (cafestol and kahweol), their impact on liver lipid biomarkers and their excretion in feces for the validation of SCGs as a safe, healthy and sustainable food ingredient with dietary fiber effect possessing high fat holding capacity.

2. Materials and methods

2.1. SCGs samples

Raw SCGs Robusta species from industrial soluble coffee production were provided by Prosol S.A (Palencia, Spain). SCGs were thermally stabilized by drying in an oven (Memmert, UM 500, Büchenbach, Germany) at 40 ºC for 9 hours until constant weight was achieved.

2.2. Safety of SCGs

2.2.1. Mycotoxins

Aflatoxin B1, enniantin B, and ochratoxin A (OTA) were analyzed by high-performance liquid chromatography–quadrupole-time of flight mass spectrometry (HPLC-QToF-MS) formed by a HPLC 1260 Infinity system (Agilent, Waldbronn, Germany) coupled to a 6545 QToF mass spectrometry detector (Agilent, Singapore) following the procedure described by Iriondo-DeHond *et al*. (2019).

2.2.2. Acute toxicity study

The acute toxicity study was performed following the directions of the OECD (Organization for Economic Co-operation and Development) Test Guidelines 425 (Up and Down Procedure). Healthy 8 weeks old nulliparous and nonpregnant female Wistar rats (Rattus norvegicus albinus), weighing 180.52 ± 6.42 g at the start of the experiment, were purchased from Charles River (Sant Cugat del Vallés, Spain). This study was approved by the Institutional Animal Ethics Committee (Reg. No. PROEX 011/17) of Community of Madrid, Spain. The animals were kept in a room with a controlled

temperature of 22 °C ± 1 °C in 12 h light-dark cycle and were housed in cages with free access to standard food (A04 Safe Diets, Augy, France) and water ad libitum. Limit test of SCGs dissolved in corn oil was performed at 2000 mg/kg b.w. by gavage as a single dose to one rat. As a control, one rat was dosed with corn oil. For the first 30 min to 4 h, rats were closely observed. If the treated rat survived, 4 additional rats per group were administered SCGs or corn oil under the same conditions. Twenty-four hours after SCGs or corn oil administration and every day for 14 days, body weight changes, signs of toxicity, behavior and mortality were observed. The last day of the study, rats were sacrificed in a gas chamber by exposure to excess carbon dioxide (CO_2). Histopathological examination was carried out in collected organs (heart, lungs, liver, kidneys, spleen, adrenal glands, sex organs and brain).

2.3. Fat, fatty acid profile and diterpenes in SCGs

Total fat content was quantified by Soxhlet extraction with petroleum ether following the procedure described by the AOAC Official Method 945.16. Results were expressed as % dry matter.

The fatty acid profile was obtained by gas chromatography (Agilent 7820A GC System equipped with Flame Ionization Detector, Waldbronn, Germany) analyses, calculated according to the ISO 12966-2:2017.

Diterpenes (cafestol and kahweol) were quantified using high liquid chromatography (Agilent 1200, Waldbronn, Germany) coupled to a quadruple triple mass spectrometer (Agilent G6410B, Waldbronn, Germany) (HPLC -QqQ). A Zorbax Eclipse XDB C18 993967-902 Agilent 150 mm x 5 μm x 4.6 mm column was used (Santa Clara, CA, USA). Two mobile phases, water and methanol in a 70% gradient, were used with a flow of 1ml/min. An atmospheric-pressure chemical ionization source (APCI) of positive polarity was used.

Samples were prepared by saponification of the dried fat with a mixture of 11.5 % KOH in ethanol water 55:45 v/v at 80 °C for 15 minutes. The unsaponifiable fraction was extracted with 1 ml of hexane. Aliquots of 0.5 ml of the solution were evaporated and resuspended in 1ml of methanol (sample diluted 1:2). For quantification, pure cafestol and kahweol standards (Cayman Chemical Company, Ann Arbor, MI, USA) were used. Each sample was prepared in duplicate and the standards were prepared following the same procedure carried out for the samples. Data were processed in the control software Masshunter Data Acquisition B.04.01 and Masshunter Qualitative Analysis B.07.00 (Waldbronn, Germany).

2.4. Bioaccesibility of lipids and diterpenes in SCGs

To study the effect of digestion on SCGs, an *in vitro* oral gastrointestinal digestion was carried out. SCGs were digested following the procedure described by Hollebeeck *et al.* (2013), with some modifications. Salivary, gastric and duodenal stages were performed in the same tube covered with aluminum foil. Approximately 1.2 g of SCGs were weighed. Conditions for each stage were: salivary stage (pH 6.9, 5 min, 3.9 U a-amylase/ml, aerobic), gastric stage (pH 2, 90 min, 71.2 U pepsin/ml, aerobic), and abiotic duodenal stage (pH 7, 150 min, 9.2 mg pancreatin and 55.2 mg bile extract/ml, aerobic). Obtained digests were centrifuged and separated in supernatant and precipitate. The content of fat and diterpenes was determined in the precipitate (non-digestible fraction also called colonic fraction) and in the supernatant following the procedure described in section 2.2. To simulate human intestinal reabsorption of bile salts, the soluble fraction was treated with cholestyramine resin (10% w/v) for 1 h at room temperature (Edwards & Slater, 2009). The resin was removed by centrifugation and gravimetric filtration.

2.5. Pilot repeated dose animal study

Male Wistar rats (average weight, 304 ± 15 g) were obtained from the Veterinary Unit at Universidad Rey Juan Carlos (URJC) (Alcorcón, Madrid, Spain) (n = 10). Animals were group-housed (2 – 4 rats/cage) in standard transparent cages (60 cm × 40 cm × 20 cm), under environmentally controlled conditions (20 °C and 60 % humidity), with a 12-hour light-dark cycle. Rats had free access to standard laboratory rat food (Harlan Laboratories Inc., Barcelona, Spain) and water. The study was approved by the Institutional Animal Ethics Committee (Reg. No. PROEX 059/18) of Community of Madrid, Spain. Rats were divided in two groups, controls (n = 6) and treated with SCGs (n = 4). Animals received SCGs (1 g/kg) or vehicle (Tween 80 in H_2O, 1.8 ml/kg) by gavage once a day from Monday to Friday for 4 consecutive weeks. A fresh SCGs solution was daily prepared. Throughout the 4 weeks, general parameters were regularly evaluated (body weight, water and food intake, and appearance of animals). At the end of the study, feces were collected for the excretion study and animals were guillotined under anesthesia with sodium pentobarbital (2 ml/kg). Internal organs were obtained for histopathological examination for safety and metabolism evaluation.

2.5.1. Metabolism study

2.5.1.1. Determination of fat and cholesterol in rat livers

Livers from control and treated rats from the pilot animal study were collected for their analysis. Liver fat was extracted by the method described by Aguilera *et al*. (2005).

Frozen rat liver samples were thawed in refrigeration to a soft consistency. Two hundred milligrams of tissue were weighed in a 15 ml plastic test tube, 250 µl of distilled water were added and the mixture was homogenized with an Ultra-Turrax (IKA, Staufen, Germany). Total fat was extracted with 1.5 ml of HCl (0.01 M), 0.1 ml of 1% $MgCl_2$ and 5 ml of hexane:isopropanol (3:2). Samples were mixed for one minute and centrifuged at 5000 rpm for 10 minutes. The liquid phase was separated to another tube and centrifuged once more at 10.000 rpm for 10 minutes. Then, the supernatant obtained was separated and concentrated in a SpeedVac Vacuum Concentrator (Thermofisher, Asheville, USA) to remove solvents for 12 hours at low temperature and under vacuum. Total fat content of livers was calculated gravimetrically. Samples from each rat liver were prepared by duplicate.

For cholesterol determination, fat from liver was dissolved in 1 ml of PBS with 50 µg of BHT/ml PBS and then, 2 ml of Triton X-110 were added. Cholesterol determination was performed spectrophotometrically using a commercial kit (Spinreact, Girona, Spain). Results were expressed in mg/dl.

2.5.1.2. Histopathological examination

Samples form the gastroinstestinal tract and liver samples were fixed in buffered formalin 10% (Panreac©, Barcelona, Spain, stabilized with methanol at pH 7) for 24 h at room temperature. Then, they were embedded in paraffin (Casa Alvarez, Madrid, Spain) using an automatic tissue processor (ASP300, Leica®, Wetzlar, Germany). Blocks were built in a block forming unit (Leica© EG1140H and Leica© EG1130, Wetzlar, Germany) and 4-micron thick sections were obtained (Leica© RM 2155, Wetzlar, Germany). Sections were deparaffinized in xylene, hydrated in alcohol and water and stained with hematoxylin and eosin (Leica© SP4040, Wetzlar, Germany). Then, sections were dehydrated through an ascending series of alcohols, cleared in xylene and finally, mounted with DPX (Nustain©, Nottingham, UK). Samples were studied qualitatively in 4 slices per animal under a Zeiss Axioskop 2 microscope equipped with the image analysis software package AxioVision 4.6 (Zeiss, Oberkochen, Germany).

2.5.2. Lipid and diterpenes excretion

Number, weight, dry weight, fat and diterpenes were determined in feces of control and treated rats collected at the end of the study following the methodology described in the section 2.3 of the present manuscript.

2.5.3. Dietary fiber effect study (gastrointestinal motility)

The evaluation of the gastrointestinal motility was performed on the 1st, 14th and 28th day of the study, by radiographic, non-invasive, in vivo methods, routinely used by the Pharmacology laboratory of the URJC (Abalo *et al.*, 2009; Cabezos *et al*., 2008). Animals were fasted overnight and SCGs at 1 g/kg were given by gavage. Thirty minutes after SCGs administration, barium contrast (a mixture of 80% barium, 5% arabic gum, 1.6% sorbitol, 0.6% citric acid and 0.8% citrate (w/v) in distilled water) was administered at 3 ml/rat, and radiographs (20 ms/shot, w/o anaesthesia) were taken 0, 1, 2, 3, 4, 6, 8 and 24h (T0 – T24) with a CS2100 (Carestream Dental, Spain) digital X-ray apparatus (60 kV, 7 mA), recorded on Carestream Dental T-MAT G/RA film (15 x 30 cm) housed in a cassette provided with regular intensifying screen and developed using an automatic processor (Kodak X-OMAT 2000, Rochester, NY, USA). For the analysis of the radiographs, after their digitalization, a semiquantitative scale was applied to each gastrointestinal region (stomach, small intestine, caecum and colorectal region) of each rat, and at each time-point, scores between 0 and 12 were obtained and represented in the corresponding motility curves (Cabezos *et al*., 2008).

2.6. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) (*in vitro* studies) or standard error (SE) (*in vivo* studies). A one- or two-way analysis of variance (ANOVA) followed by Tukey's or Bonferroni's test (gastrointestinal motility analysis) for mean comparisons was used to highlight the significant differences among samples. Student T test was performed to compare means between two groups. Values of $p < 0.05$ (*in vitro* studies) and $p < 0.1$ (*in vivo* studies) were considered statistically significant. Statistical analyses were performed using SPSS Statistics 24 (IBM, Armonk, NY, USA) and GraphPad Prism program version 5.01 (GraphPad software, San Diego, CA, USA, for the gastrointestinal motility analysis).

3. Results and discussion

3.1. Safety of SCGs

Coffee is susceptible to contamination by mycotoxins, toxic compounds that result from fungal secondary metabolism under certain conditions, which cause different toxicological effects in humans (Afsah-Hejri *et al*., 2013). Studies carried out by Garcia-Moraleja *et al*. (2015) indicate that aflatoxin B1, enniantin B and OTA are the most frequent mycotoxins present in coffee beverages. Neither aflatoxin B1 nor enniantin B were detected but OTA levels in SCGs were 2.31 ± 0.07 µg/kg. Commission Regulation

(EC) No 123/2005 defined OTA limits as 5 μg/kg for roasted coffee and 10 μg/kg for soluble coffee (European Commission, 2005). Values of OTA obtained for instant Robusta SCGs used in this study were below the limit stablished by the European legislation.

Considering that OTA causes major health risks such as hepatotoxicity (Kőszegi & Poór, 2016), an acute toxicity study in rats was carried out. Single oral administration of the SCGs at a dose of 2000 mg/kg b.w. resulted in no visible signs of toxicity, abnormal behavior or mortality. Organ weights are shown in Table 1 and no significant differences ($p > 0.05$) were found among groups. Intake of an acute dose of SCGs (2000 mg/ kg b.w.) did not cause significant changes in histological parameters of vital organs (data not shown). To the best of our knowledge, mycotoxins and acute toxicity have been analyzed in this coffee byproduct for the first time.

Table 1. Organ weights in female rats included in the control group and in the group exposed to the limit dose of 2000 mg/kg b.w.

Organs	Control	Treatment
Heart	1.02 ± 0.23 [a]	0.90 ± 0.07 [a]
Lungs	1.90 ± 0.25 [a]	1.56 ± 0.23 [a]
Liver	9.72 ± 1.21 [a]	9.94 ± 1.25 [a]
Kidneys	2.08 ± 0.11 [a]	2.00 ± 0.19 [a]
Spleen	0.62 ± 0.15 [a]	0.60 ± 0.10 [a]
Thymus	0.66 ± 0.15 [a]	0.58 ± 0.13 [a]
Adrenal glands	0.16 ± 0.09 [a]	0.18 ±0.08 [a]
Uterus	1.52 ± 0.67 [a]	1.36 ± 0.18 [a]
Brain	1.80 ± 0.18 [a]	1.70 ± 0.20 [a]

Values represent means ± SD (n = 5). Different letters indicate significant differences (T test, $p < 0.05$).

3.2. In vitro bioaccesibility of lipids and diterpens composing SCGs

Total fat content and the fatty acid profile of instant Robusta SCGs are shown in Table 2. Fat content of SCGs was 21.79 %, which is in accordance with that previously described by other authors for SCGs from soluble coffee (Martinez-Saez *et al.*, 2017; Pujol *et al.*, 2013). Palmitic (62.58 mg/g), stearic (14.76 mg/g), oleic (20.38 mg/g), linoleic (87.36 mg/g) and arachidic (6.68 mg/g) acids were found in the SCGs sample

being the 56 % of the total fatty acids unsaturated. The presence of higher amounts of polyunsaturated fatty acids (PUFA) than saturated fatty acids (SFA) in oil is recognized as being more positive for human health (Acevedo *et al.*, 2013). In SCGs, PUFA were only represented by linoleic acid. The PUFA/SFA ratio of SCGs was 1.038. Oils with PUFA/SFA ratios > 1 have been reported to be less atherogenic and thrombogenic than those with ratio < 1 due to the potential favorable reduction of serum cholesterol and atherosclerosis and prevention of heart diseases (Campos-Vega *et al.*, 2015).

With regard to diterpene content, cafestol and kahweol levels in SCGs were 3.09 mg/g and 64.15 µg/g, respectively. Amounts of cafestol in SCGs are in line with values obtained by Acevedo *et al*. (2013) but kahweol levels were lower (1.64 mg/g). However, kahweol values obtained in this study are similar to those present in Robusta coffee beans (De Roos *et al*., 1998). In addition, differences in values between studies are possible since levels of diterpenes in coffee beans depend on the species and storage conditions as kahweol and cafestol are sensitive to acids, heat and light (Acevedo *et al.*, 2013).

After *in vitro* digestion, half of the total fat from SCGs was bioaccessible and half was excreted in the insoluble fraction (Table 2). It has been reported that SCGs possess high oil-holding capacity, which is possibly due to the presence of lignin (Ballesteros *et al*., 2014). This capacity is a very desirable parameter for the functionality of a dietary fiber (Ballesteros *et al*., 2014). The 77 % of unsaturated fatty acids remained bioaccessible after *in vitro* digestion including linoleic (54.01 mg/g) and oleic acids (16.42 mg/g), as the most abundant, followed by the SFA palmitic acid (15.18 mg/g). With regard to diterpenes, low amounts of kahweol (7.09 µg/g) and cafestol (414.39 µg/g) were bioaccessible (Table 2). Knowledge about the absorption and metabolism of coffee diterpenes is limited. However, data obtained from animal studies treated with ^{3}H-labeled cafestol have shown that cafestol is efficiently absorbed and partially metabolized by the gut, further metabolized by the liver, and then excreted into the bile (Van Cruchten *et al*., 2010). It is known that cafestol is a cholesterol-raising compound in coffee beans that can directly regulate expression of genes involved in cholesterol metabolism (Ricketts *et al.*, 2007). A very recent randomized crossover clinical trial has reported that unfiltered coffee consumption increased concentrations of serum lipids and total cholesterol levels in a healthy adult population, possibly due to cafestol (Eren & Besler, 2019). According to these results, most of the unhealthy lipid compounds forming instant SCGs may be bound to the dietary fiber and excreted in feces.

Table 2. Total fat (%), fatty acid profile (mg/g sample and % of total) and diterpenes (μg/g sample) of spent coffee grounds (SCGs) and their bioaccessible and excreted fraction obtained after *in vitro* oral-gastrointestinal digestion.

Analysis	**SCGs**		**Bioaccessible fraction**		**Fiber fraction**	
Total fat (%)	21.79 ± 0.23 ^{b}		11.87 ± 1.66 ^{a}		14.21 ± 0.82 ^{a}	
Fatty acid	**(mg/g)**	**(%)**	**(mg/g)**	**(%)**	**(mg/g)**	**(%)**
C14:0	0.21 ± 0.01 ^{c}	0.11	0.12 ± 0.01 ^{a}	0.13	0.19 ± 0.01 ^{b}	0.17
C15:0	0.09 ± 0.00 ^{b}	0.05	0.06 ± 0.00 ^{a}	0.06	0.11 ± 0.01 ^{c}	0.10
C16:0	62.58 ± 2.38 ^{c}	31.58	15.18 ± 0.94 ^{a}	15.59	41.56 ± 0.74 ^{b}	37.87
C16:1n7	0.14 ± 0.01 ^{a}	0.07	0.39 ± 0.03 ^{c}	0.40	0.21 ± 0.01 ^{b}	0.19
C17:0	0.17 ± 0.01 ^{b}	0.09	0.11 ± 0.01 ^{a}	0.11	0.28 ± 0.01 ^{c}	0.26
C18:0	14.76 ± 0.56 ^{b}	7.45	3.67 ± 0.30 ^{a}	3.77	11.67 ± 0.16 ^{c}	10.64
C18:1n7c	0.77 ± 0.03 ^{b}	0.39	0.88 ± 0.06 ^{c}	0.90	0.54 ± 0.03 ^{a}	0.50
C18:1n9c	20.38 ± 0.84 ^{c}	10.28	16.42 ± 1.23 ^{b}	16.84	10.62 ± 0.43 ^{a}	9.67
C18:2n6c	87.36 ± 3.73 ^{c}	44.07	54.01 ± 4.82 ^{b}	55.34	36.85 ± 0.99 ^{a}	33.57
C18:3n3	1.75 ± 0.09 ^{c}	0.88	1.03 ± 0.09 ^{b}	1.05	0.76 ± 0.03 ^{a}	0.69
C18:3n6	n.d.	0.00	0.08 ± 0.01 ^{b}	0.08	0.04 ± 0.01 ^{a}	0.04
C20:0	6.68 ± 0.26 ^{c}	3.37	1.23 ± 0.10 ^{a}	1.26	3.90 ± 0.07 ^{b}	3.55
C20:1n9	0.83 ± 0.04 ^{c}	0.42	0.44 ± 0.03 ^{b}	0.45	0.32 ± 0.01 ^{a}	0.30
C20:2n6	0.12 ± 0.01 ^{b}	0.06	0.17 ± 0.01 ^{c}	0.17	0.09 ± 0.01 ^{a}	0.08
C20:3n6	0.21 ± 0.01 ^{c}	0.10	0.04 ± 0.00 ^{a}	0.04	0.13 ± 0.00 ^{b}	0.12
C20:4n6	n.d.	0.00	2.59 ± 0.37 ^{b}	2.65	0.99 ± 0.10 ^{a}	0.90
C20:5n3	0.14 ± 0.01 ^{c}	0.07	0.11 ± 0.01 ^{b}	0.11	0.06 ± 0.01 ^{a}	0.05
C21:0	n.d.	0.00	0.10 ± 0.01 ^{b}	0.10	0.05 ± 0.01 ^{a}	0.04
C22:0	0.96 ± 0.03 ^{c}	0.49	0.20 ± 0.02 ^{a}	0.21	0.59 ± 0.01 ^{b}	0.54
C22:1n9	0.21 ± 0.01 ^{b}	0.11	0.10 ± 0.01 ^{a}	0.11	0.10 ± 0.01 ^{a}	0.09
C22:4n6	n.d.	0.00	0.11 ± 0.02 ^{b}	0.11	0.05 ± 0.00 ^{a}	0.04
C22:5n3	n.d.	0.00	0.20 ± 0.03 ^{b}	0.21	0.10 ± 0.01 ^{a}	0.09
C22:6n3	n.d.	0.00	0.10 ± 0.01 ^{b}	0.10	0.04 ± 0.01 ^{a}	0.04
C23:0	0.25 ± 0.00 ^{c}	0.13	0.06 ± 0.01 ^{a}	0.07	0.17 ± 0.01 ^{b}	0.15
C24:0	0.58 ± 0.02 ^{c}	0.29	0.13 ± 0.02 ^{a}	0.13	0.35 ± 0.00 ^{b}	0.32
SFA (%)	43.54 ± 0.11 ^{c}	-	21.43 ± 0.75 ^{a}		53.64 ± 0.49 ^{b}	
MUFA (%)	11.27 ± 0.01 ^{b}	-	18.70 ± 0.22 ^{c}		10.74 ± 0.21 ^{a}	
PUFA (%)	45.19 ± 0.11 ^{b}	-	59.87 ± 0.97 ^{c}		35.62 ± 0.29 ^{a}	
Diterpenes (μg/g)						
Cafestol	3095.39 ± 518.81 ^{b}		414.39 ± 25.80 ^{a}		1029.90 ± 55.51 ^{a}	
Kahweol	64.19 ± 9.35 ^{b}		7.09 ± 1.68 ^{a}		22.50 ± 0.34 ^{a}	

N.d., non-detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Results are expressed as mean ± SD. Different letters indicate significant differences (Tuckey test, $p < 0.05$).

3.3. In vivo metabolism and excretion of lipids composing SCGs

No adverse clinical signs (changes in body weight, food and water intake, hydration and animal behavior) or mortality were observed in animals treated with SCGs (1 g/kg b.w.) for 28 days. Altogether, the results obtained in the study seem to indicate that the administration of SCGs at 1 g/kg b.w. for 4 weeks was not noxious to the animals. During the pilot study, changes in body weight and food and water intakes were comparable in both groups throughout the experiment ($p > 0.05$). The mean weight of control and treated animals at the beginning of the study was 312 ± 17 g and 298 ± 9 g, respectively. Throughout the four weeks of the pilot study, there was an increase in weight in both groups, without showing significant differences in this parameter ($p > 0.05$) due to the intake of SCGs. Food and water intake of the control group was 23 ± 4 g/day/rat and 34 ± 9 ml/day/rat, and that of the treated group was 20 ± 3 g/day/rat and 30 ± 8 ml/day/rat. No statistically significant differences were observed between groups ($p > 0.05$).

At the end of the study, vital organs were weighed and no significant changes were observed between control and treated rats ($p > 0.05$). In addition, organs were subjected to histopathological examination and no significant damage was observed in their architecture.

The liver is a major organ for the synthesis, metabolism, storage and distribution of lipids and it plays an essential role in regulating energy metabolism (Tirosh, 2018). Thus, liver from treated and control rats was analyzed after the pilot study. Treatment of animals with instant SCGs for 28 days did not cause significant differences ($p > 0.1$) in liver weight (Figure 1A). On the contrary, fat content in treated rats was significantly lower than that for control rats ($p < 0.1$) although no lipid droplets or histological damage was observed in the liver (Figure 1D) and cholesterol levels between control rats and those treated with SCGs did not differ significantly ($p > 0.1$) (Figure 1C).

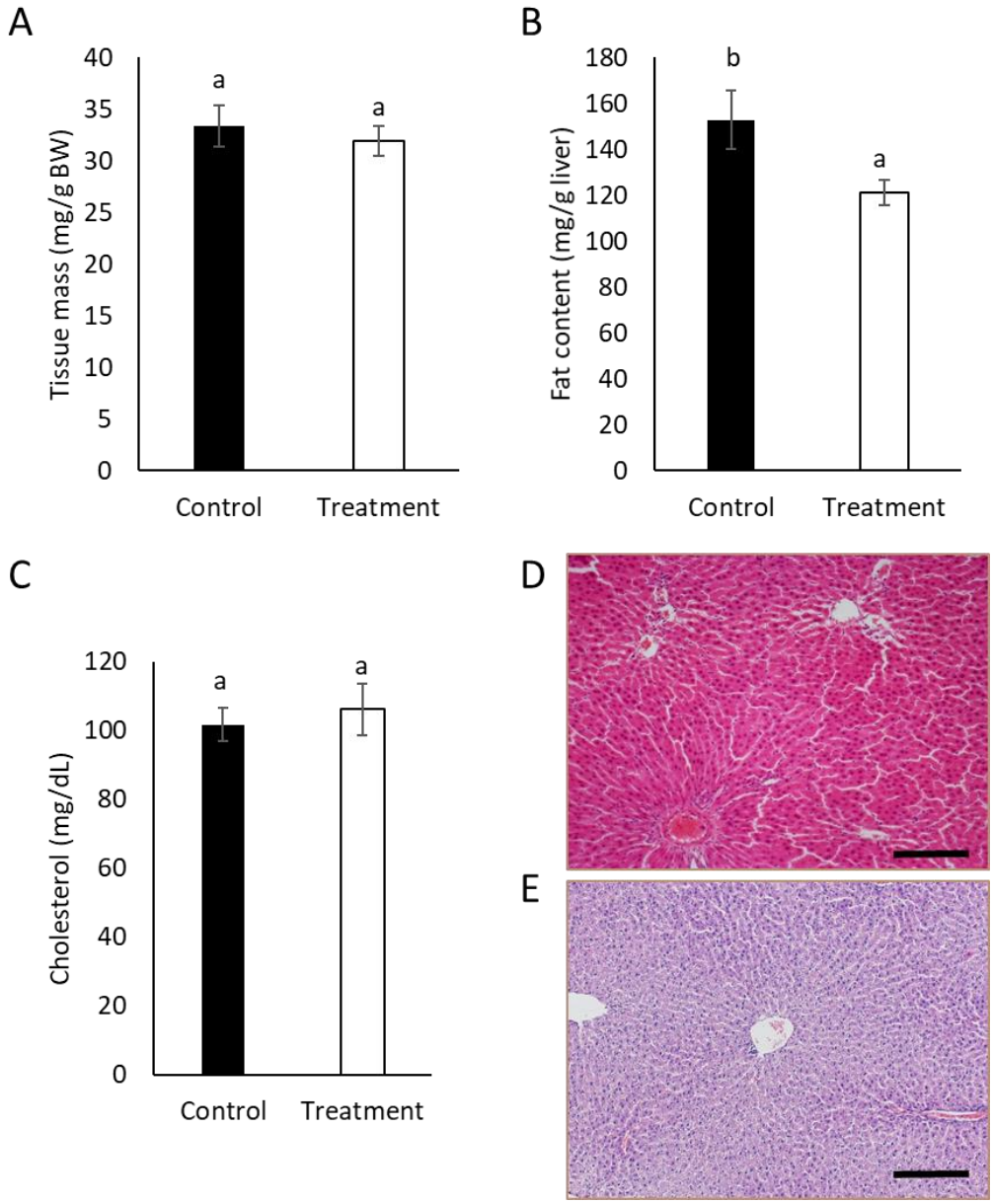


Figure 1. (A) Tissue mass (g/g BW), (B) fat content (mg/g liver) and (C) cholesterol content (mg/dL) of livers from control and treated animals. Data expressed as means ± SE. Different letters indicate significant differences between groups (T test, $p < 0.1$). Hematoxilin-eosin staining of formalin-fixed samples of the liver of a control rat (D) and a rat treated with SCGs (E). Samples were obtained on the 29th day. Bar 200 μm.

Nonalcoholic fatty liver disease is related to obesity and metabolic syndrome and it is characterized by excess of fat deposition in the liver, which leads to an increased risk of type 2 diabetes mellitus, hyperlipidemia and insulin resistance (Tirosh, 2018). No previous studies have reported the effect of SCGs on liver health, but there is increasing evidence regarding the protective effects of coffee consumption in the development of liver disease due to hepatitis B and C, nonalcoholic fatty liver disease and alcoholic liver disease (Nieber, 2017). The main responsible compounds proposed for the hepatoprotective effects of coffee are caffeine, phenolic compounds and melanoidins. These compounds are responsible for the antioxidant effects at the hepatic level that prevent free radical tissue damage by reducing reactive oxygen species, which play a central part in the inflammation processes characterizing nonalcoholic fatty liver disease and other liver diseases (Grosso *et al*., 2017). Since these compounds are also present in SCGs (Martinez-Saez *et al*., 2017), this byproduct may be a good candidate for the treatment of metabolic syndrome. Further studies, using specific models, are needed to confirm its capacity to prevent hepatic steatosis.

Figure 2 shows weight, number and fat content of feces collected the 28th day of the study of control and treated rats. Wet and dry weight and number of feces were significantly increased ($p < 0.1$) in rats treated with SCGs when compared to the control group. In addition, feces of rats treated with SCGs showed a significant higher fat content ($p < 0.1$) than feces of the control group, suggesting higher excretion of fat. These results are in accordance with that obtained in the *in vitro* oral gastrointestinal digestion assays (Table 2), which also showed a tendency of higher fat excretion. In contrast, no cafestol or kahweol were detected in feces.

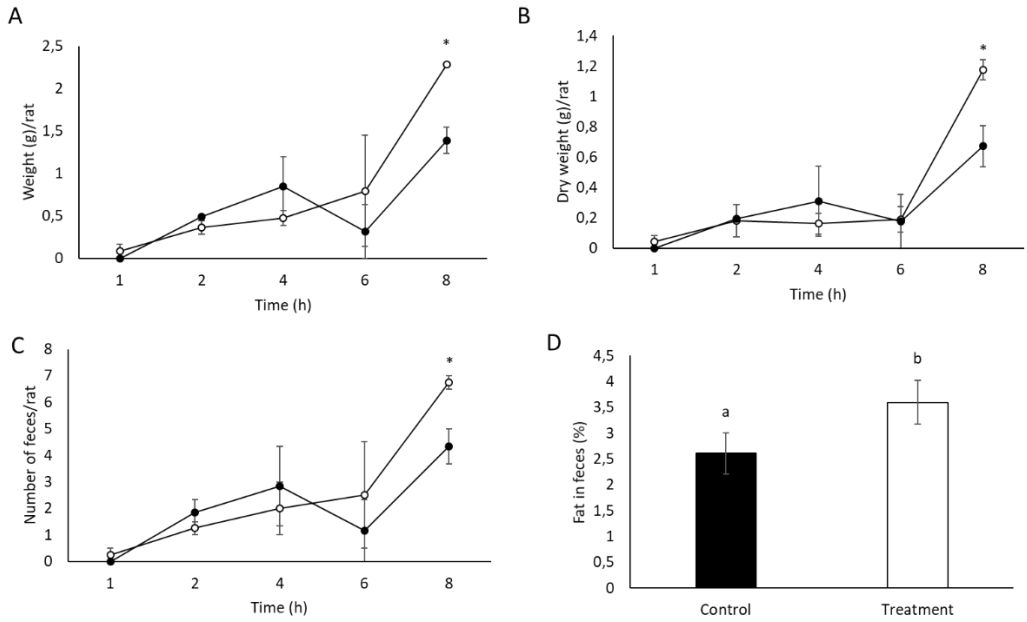


Figure 2. Weight (A), dry weight (B), number (C) and fat content (D) in feces collected the 28th day of the study during the last X-ray session. Data represent the means ± SE. Asterisks indicate significant differences between groups at each hour (T test, $p < 0.1$). In the analysis of fat content of feces, different letters denote significant differences (T test, $p < 0.1$).

3.4. Gastrointestinal motility

Tissue samples of organs from the gastrointestinal tract of treated rats are shown in Figure 3. As can be observed, the different gastrointestinal regions showed normal morphology suggesting no significant damage caused by the repeated intake of SCGs (1 g/ kg b.w.) during 28 days.

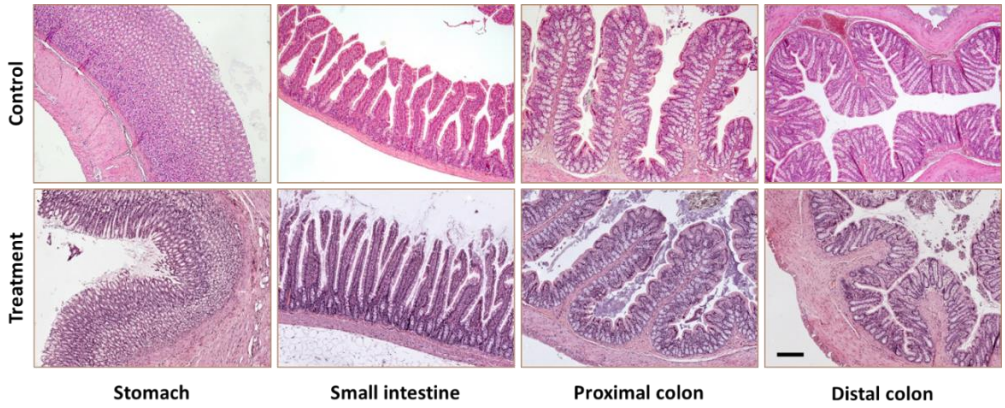


Figure 3. Histological staining showing stomach, small intestine, proximal and distal colon architecture (hematoxilin-eosin staining of formalin-fixed samples) from control rats and rats treated with SCGs. Samples were obtained on the 29th day. Bar 200 μm.

The fiber effect of SCGs was studied using radiographic analyses performed on the 1st, 14th and 28th day of the study. Results of the radiographic study carried out on the 1st day of the study are shown in Figure 4. Control rats showed normal motor function in all gastrointestinal regions and radiographic sessions. SCGs did not significantly alter the normal progressive gastric emptying in any of the X-ray sessions compared to the control group ($p > 0.05$). In contrast, SCGs significantly accelerated ($p < 0.05$) intestinal transit during the first X-ray session. Results of the semiquantitative analysis of the small intestine showed a filling phase (0 – 1 h), a plateau (1 – 3 h), and a progressive emptying phase (3 – 8 h) in the control group (Figure 4B). However, animals treated with SCGs showed a filling maximum at 2 hours that was significantly higher than that of control rats ($p < 0.005$). With regard to the caecum, significant differences were observed ($p < 0.001$), since it took 4 hours for the caecum of the control animals to be completely filled, whereas that of the treated animals was already filled in 2 hours (Figure 4B).

Considering the colorectum curve, the control group began to form fecal pellets 4 hours after barium administration and reached the maximum score at 8 hours of the radiographic session. However, SCGs significantly accelerated fecal pellet formation at 3 and 4 hours after contrast administration ($p < 0.001$ and $p < 0.05$, respectively).

On the contrary, on X-rays taken on the 14th and 28th day of treatment, intestinal motor function overlapped with that of controls (Figures 4C and 4D). This suggests that, upon chronic treatment, tolerance may develop to the intestinal stimulating effect of SCGs (Muller-Lissner *et al*., 2005). Importantly, no sign of impaired motility was found in any

animal. To the best of our knowledge, no previous studies have reported the effect of SCGs on intestinal motility.

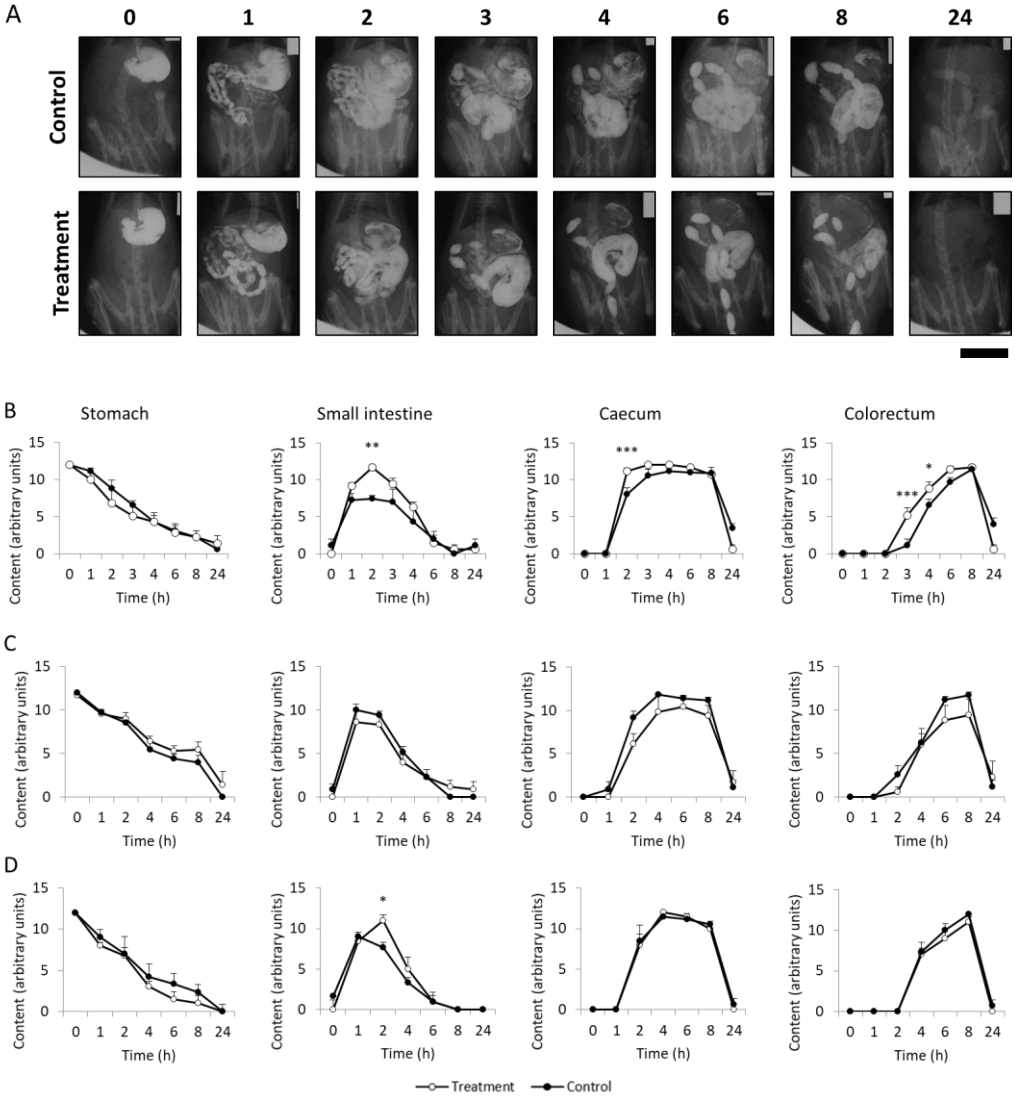


Figure 4. (A) Radiological images of X-rays taken on the 1st day of treatment of control and rats treated with SCGs taken immediately and 1, 2, 3, 4, 6, 8 and 24 h after barium administration. Scale bar = 3 cm. Radiological analysis of X-rays taken on the 1st (B), 14th (C) and 28th (D) day of treatment according to Cabezos *et al.* (2008). Data represent the mean ± SE. * ($p < 0.05$), ** ($p < 0.005$), *** ($p < 0.001$) (two-way ANOVA followed by Bonferroni test).

Data hereby reported support the dietary fiber effect of SCGs and their potential for modulating lipid metabolism which may be associated to their fat holding capacity. Other gastrointestinal health-promoting properties have been attributed to SCGs. Lopez-Barrera *et al*. (2016) reported that SCGs can be fermented by colon microbiota producing short chain fatty acids (SCFAs) with anti-inflammatory properties. SCFAs generated after fermentation of SCGs had the ability to suppress NO production and inhibited inflammatory mediators (cytokines IL-10, CCL-17, CXCL9, IL-1b and IL-5) in murine macrophage cells (Campos-Vega *et al*., 2015; López-Barrera *et al*., 2016)(R. Campos-Vega et al., 2015)(R. Campos-Vega et al., 2015)(R. Campos-Vega et al., 2015)(R. Campos-Vega et al., 2015)(R. Campos-Vega et al., 2015)(R. Campos-Vega et al., 2015)(R. Campos-Vega et al., 2015)(R. Campos-Vega et al., 2015)(R. Campos-Vega et al., 2015)(R. Campos-Vega et al., 2015). The *in vivo* effects of SCFAs on colonic motility have been controversial. However, it has been shown that luminal administration of SCFAs (10 - 200 mM) stimulates colonic motility (Fukumoto *et al*., 2003). Lopez-Barrera *et al*. (2016) reported the generation of SCFAs in concentrations higher than 10 mM during colonic fermentation of SCGs from medium and dark roasted coffee beans. Results obtained in the present research regarding gastrointestinal motility might be influenced by SCFAs released during SCGs fermentation.

4. Conclusions

Acute and repeated treatments of Wistar rats with SCGs were not noxious to the animals. Most of the SFA and diterpenes forming instant SCGs were not bioaccesible after *in vitro* digestion. Results seem to indicate that they may be bound to the SCGs dietary fiber and excreted in feces reducing fat accumulation in liver. These results suggest that SCGs might be used as a sustainable, safe and healthy food ingredient for preventing hepatic steatosis and treating metabolic syndrome. Further studies in humans should be conducted to confirm this hypothesis.

Acknowledgments: A. Iriondo-DeHond is a fellow of the FPI predoctoral program of MINECO (BES-2015-072191). E. Guisantes-Batan thanks the Social European Fund and Junta de Comunidades de Castilla-La Mancha for the co-finance of his contract (OE-154). Authors thank Maica Merino and Lorena Blanco for their technical assistance with the repeated dose study at URJC and Dr. Elena Hevia and Fernando Nuñez from the Animal facility of the CBMSO, CSIC, for their help in the acute toxicity study. We are grateful to the Analysis Service Unit facilities of ICTAN for the analysis of Chromatography and Mass Spectrometry. L. Blanco had a contract by Consejería de Educación, Juventud y Deporte from Comunidad de Madrid and Fondo Social Europeo (PEJ15/BIO/TL-0580).

Funding: This research was funded by the SUSCOFFEE (AGL2014-57239-R) Project.

Author Contributions: Conceptualization and investigation, M.D.d.C. and R.A.; Methodology, M.I.S.A., S.S.-F., J.A.U., S.G.A., R.A., and M.D.d.C.; Data Curation, A.I.-D., F.S.C., B.F.-G., E.G.-B., G.V., and L.L.-G.; Formal Analysis, A.I.-D., F.S.C., B.F.-G., E.G.-B., G.V., L.L.-G. M.I.S.A., S.S.-F., J.A.U., S.G.A., R.A., and M.D.d.C.; Writing —Original Draft Preparation, A.I.-D. and M.D.d.C.; Writing—Review & Editing, A.I.-D., F.S.C., B.F.-G., E.G.-B., G.V., L.L.-G., M.I.S.A., S.S.-F., J.A.U., S.G.A., R.A., and M.D.d.C.; Supervision, R.A. and M.D.d.C.; Project Administration, M.D.d.C.; Funding Acquisition, M.D.d.C. and R.A.

Conflicts of Interest: The authors declare no conflict of interest.

References

Abalo, R., Cabezos, P. A., López-Miranda, V., Vera, G., González, C., Castillo, M., … Martín, M. I. (2009). Selective lack of tolerance to delayed gastric emptying after daily administration of WIN 55,212-2 in the rat. *Neurogastroenterology and Motility*, *21*(9), 22–24. https://doi.org/10.1111/j.1365-2982.2009.01315.x.

Acevedo, F., Rubilar, M., Scheuermann, E., Cancino, B., Uquiche, E., Garcés, M., … Shene, C. (2013). Spent coffee grounds as a renewable source of bioactive compounds. *Journal of Biobased Materials and Bioenergy*, *7*(3), 420–428. https://doi.org/10.1166/jbmb.2013.1369.

Afsah-Hejri, L., Jinap, S., Hajeb, P., Radu, S., & Shakibazadeh, S. (2013). A Review on Mycotoxins in Food and Feed: Malaysia Case Study. *Comprehensive Reviews in Food Science and Food Safety*, *12*(6), 629–651. https://doi.org/10.1111/1541-4337.12029.

Aguilera, C. M., Ramirez-Tortosa, C. L., Quiles, J. L., Yago, M. D., Martínez-Burgos, M. A., Martínez-Victoria, E., … Ramirez-Tortosa, M. C. (2005). Monounsaturated and ω-3 but not ω-6 polyunsaturated fatty acids improve hepatic fibrosis in hypercholesterolemic rabbits. *Nutrition*, *21*(3), 363–371. https://doi.org/10.1016/J.NUT.2004.06.029.

Ballesteros, L. F., Teixeira, J. a., & Mussatto, S. I. (2014). Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. *Food and Bioprocess Technology*, *7*(12), 3493–3503. https://doi.org/10.1007/s11947-014-1349-z.

Cabezos, P. A., Vera, G., Castillo, M., Fernández-Pujol, R., Martín, M. I., & Abalo, R. (2008). Radiological study of gastrointestinal motor activity after acute cisplatin in the rat. Temporal relationship with pica. *Autonomic Neuroscience*, *141*(1), 54–65. https://doi.org/10.1016/j.autneu.2008.05.004.

Calligaris, S., Munari, M., Arrighetti, G., & Barba, L. (2009). Insights into the physicochemical properties of coffee oil. *European Journal of Lipid Science and Technology*, *111*(12), 1270–1277. https://doi.org/10.1002/ejlt.200900042.

Campos-Vega, R., Vázquez-Sánchez, K., López-Barrera, D., Loarca-Piña, G., Mendoza-Díaz, S., & Oomah, B. D. (2015). Simulated gastrointestinal digestion and *in vitro* colonic fermentation of spent coffee (Coffea arabica L.): Bioaccessibility and intestinal permeability. *Food Research International, 77*, 156–161. https://doi.org/10.1016/j.foodres.2015.07.024.

Campos-Vega, R., Loarca-Piña, G., Vergara-Castañeda, H., & Oomah, B. D. (2015). Spent coffee grounds: A review on current research and future prospects. *Trends in Food Science & Technology, 45*(1), 24–36. https://doi.org/10.1016/j.tifs.2015.04.012.

De Roos, B., Meyboom, S., Kosmeijer-Schuil, T. G., & Katan, M. B. (1998). Absorption and urinary excretion of the coffee diterpenes cafestol and kahweol in healthy ileostomy volunteers. *Journal of Internal Medicine, 244*(6), 451–460. https://doi.org/10.1046/j.1365-2796.1998.00386.x.

del Castillo, M. Dolores, Fernandez-Gomez, B., Martinez-Saez, N., Iriondo-DeHond, A., & Mesa, M. D. (2019). Coffee By-Products. In A. Farah (Ed.), Coffee: Chemistry, Quality and Health Implications. Oxfordshire: Royal Society of Chemistry. UK; ISBN 9781782620044.

del Castillo, M.D., Martinez-Saez, N., & Ullate, M. (2014). Healthy Bakery Products with High Level of Dietary Antioxidant Fibre. International Patent WO2014/128320Al, Spain.

Edwards, A. D., & Slater, N. K. H. (2009). Protection of live bacteria from bile acid toxicity using bile acid adsorbing resins. *Vaccine, 27*(29), 3897–3903. https://doi.org/10.1016/j.vaccine.2009.04.006.

Eren, F. H., & Besler, H. T. (2019). A 4-week consumption of light or dark roast unfiltered (Turkish) coffee affects cardiovascular risk parameters of homocysteine and cholesterol concentrations in healthy subjects: a randomized crossover clinical trial. *Progress in Nutrition, 21*(1), 164–173. https://doi.org/10.23751/pn.v21i1.7662.

European Commission. (2005). COMMISSION REGULATION (EC) No 123/2005 of 26 January 2005 amending Regulation (EC) No 466/2001 as regards ochratoxin A. *Official Journal of the European Union, 2005*(123), 2004–2006. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R0123&qid=1478028660364&from=ES.

Fukumoto, S., Tatewaki, M., Yamada, T., Fujimiya, M., Mantyh, C., Voss, M., … Takahashi, T. (2003). Short-chain fatty acids stimulate colonic transit via intraluminal 5-HT release in rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 284*(5), R1269–R1276. https://doi.org/10.1152/ajpregu.00442.2002.

Garcia-Moraleja, A., Font, G., Manes, J., & Ferrer, E. (2015). Analysis of mycotoxins in coffee and risk assessment in Spanish adolescents and adults. Food and Chemical Toxicology, 86, 225–233. https://doi.org/10.1016/j.fct.2015.10.014.

George, S. E., Ramalakshmi, K., & Rao, L. J. M. (2008). A perception on health benefits of coffee. *Critical Reviews in Food Science and Nutrition, 48*(5), 464–486. https://doi.org/10.1080/10408390701522445.

Grosso, G., Godos, J., Galvano, F., & Giovannucci, E. L. (2017). Coffee, Caffeine, and Health Outcomes: An Umbrella Review. *Annual Review of Nutrition, 37*(1), 131–156. https://doi.org/10.1146/annurev-nutr-071816-064941.

Hollebeeck, S., Borlon, F., Schneider, Y.-J., Larondelle, Y., & Rogez, H. (2013). Development of a standardised human in vitro digestion protocol based on macronutrient digestion using response surface methodology. *Food Chemistry, 138*(2–3), 1936–1944. https://doi.org/10.1016/j.foodchem.2012.11.041.

Iriondo-DeHond, A., Aparicio García, N., Velazquez Escobar, F., San Andres, M. I., Sanchez-Fortun, S., Blanch, G. P., … del Castillo, M. D. (2019). Validation of coffee by-products as novel food ingredients. *Innovative Food Science and Emerging Technologies, 51*, 194–204. https://doi.org/10.1016/j.ifset.2018.06.010.

Kőszegi, T., & Poór, M. (2016). Ochratoxin a: Molecular interactions, mechanisms of toxicity and prevention at the molecular level. *Toxins, 8*(4), 1–25. https://doi.org/10.3390/toxins8040111.

Kovalcik, A., Obruca, S., & Marova, I. (2018). Valorization of spent coffee grounds: A review. *Food and Bioproducts Processing, 110*, 104–119. https://doi.org/10.1016/j.fbp.2018.05.002.

López-Barrera, D. M., Vázquez-Sánchez, K., Loarca-Piña, M. G. F., & Campos-Vega, R. (2016). Spent coffee grounds, an innovative source of colonic fermentable compounds, inhibit inflammatory mediators *in vitro*. *Food Chemistry, 212*, 282–290. https://doi.org/10.1016/j.foodchem.2016.05.175.

Martinez-Saez, N., Tamargo García, A., Domínguez Pérez, I., Rebollo-Hernanz, M., Mesías, M., Morales, F. J., … del Castillo, M. D. (2017). Use of spent coffee grounds as food ingredient in bakery products. *Food Chemistry, 216*, 114–122. https://doi.org/10.1016/j.foodchem.2016.07.173.

Muller-Lissner, S. A., Kamm, M. A., Scarpignato, C., & Wald, A. (2005). Myths and Misconceptions About Chronic Constipation. *American Journal of Gastroenterology, 100*, 232–242. https://doi.org/10.1111/j.1572-0241.2004.40885.x.

Murthy, P. S., & Madhava Naidu, M. (2012). Sustainable management of coffee industry by-products and value addition - A review. *Resources, Conservation and Recycling, 66*, 45 - 58. https://doi.org/10.1016/j.resconrec.2012.06.005.

Mussatto, S. I., Carneiro, L. M., Silva, J. P. A., Roberto, I. C., & Teixeira, J. A. (2011). A study on chemical constituents and sugars extraction from spent coffee grounds. *Carbohydrate Polymers*, *83*(2), 368–374. https://doi.org/10.1016/j.carbpol.2010.07.063.

Nieber, K. (2017). The Impact of Coffee on Health Author Pharmacokinetics and Mode of Action Bioactive Components in Coffee. *Planta Med*, *83*(1), 1256–1263.

Padmapriya, R., Tharian, J., & Thirunalasundari, T. (2013). Coffee waste management-An overview. *International Journal of Current Science*, *9*, 83–91. Retrieved from http://www.currentsciencejournal.info/issuespdf/Coffee waste12.pdf.

Pujol, D., Liu, C., Gominho, J., Olivella, M. À., Fiol, N., Villaescusa, I., & Pereira, H. (2013). The chemical composition of exhausted coffee waste. *Industrial Crops and Products*, *50*, 423–429. https://doi.org/10.1016/j.indcrop.2013.07.056.

Ricketts, M.-L., Boekschoten, M. V., Kreeft, A. J., Hooiveld, G. J. E. J., Moen, C. J. A., Müller, M., … Moore, D. D. (2007). The Cholesterol-Raising Factor from Coffee Beans, Cafestol, as an Agonist Ligand for the Farnesoid and Pregnane X Receptors. *Molecular Endocrinology*, *21*(7), 1603–1616. https://doi.org/10.1210/me.2007-0133.

Silva, V. M., Vieira, G. S., & Hubinger, M. D. (2014). Influence of different combinations of wall materials and homogenisation pressure on the microencapsulation of green coffee oil by spray drying. *Food Research International*, *61*, 132–143. https://doi.org/10.1016/J.FOODRES.2014.01.052.

Simões, J., Nunes, F. M., Domingues, M. R., & Coimbra, M. A. (2013). Extractability and structure of spent coffee ground polysaccharides by roasting pre-treatments. *Carbohydrate Polymers*, *97*(1), 81–89. https://doi.org/10.1016/j.carbpol.2013.04.067.

Tirosh, O. (2018). Hypoxic Signaling and Cholesterol Lipotoxicity in Fatty Liver Disease Progression. *Oxidative Medicine and Cellular Longevity*, *2018*, 1–15. https://doi.org/10.1155/2018/2548154.

Van Cruchten, S. T. J., De Waart, D. R., Kunne, C., Hooiveld, G. J. E. J., Boekschoten, M. V., Katan, M. B., … Witkamp, R. F. (2010). Absorption, distribution, and biliary excretion of cafestol, a potent cholesterol-elevating compound in unfiltered coffees, in mice. *Drug Metabolism and Disposition*, *38*(4), 635–640. https://doi.org/10.1124/dmd.109.030213.

Vázquez-Sánchez, K., Martinez-Saez, N., Rebollo-Hernanz, M., del Castillo, M. D., Gaytán-Martínez, M., & Campos-Vega, R. (2018). In vitro health promoting properties of

antioxidant dietary fiber extracted from spent coffee (*Coffee arabica* L.) grounds. *Food Chemistry, 261*, 253–259. https://doi.org/10.1016/j.foodchem.2018.04.064.

CHAPTER 4

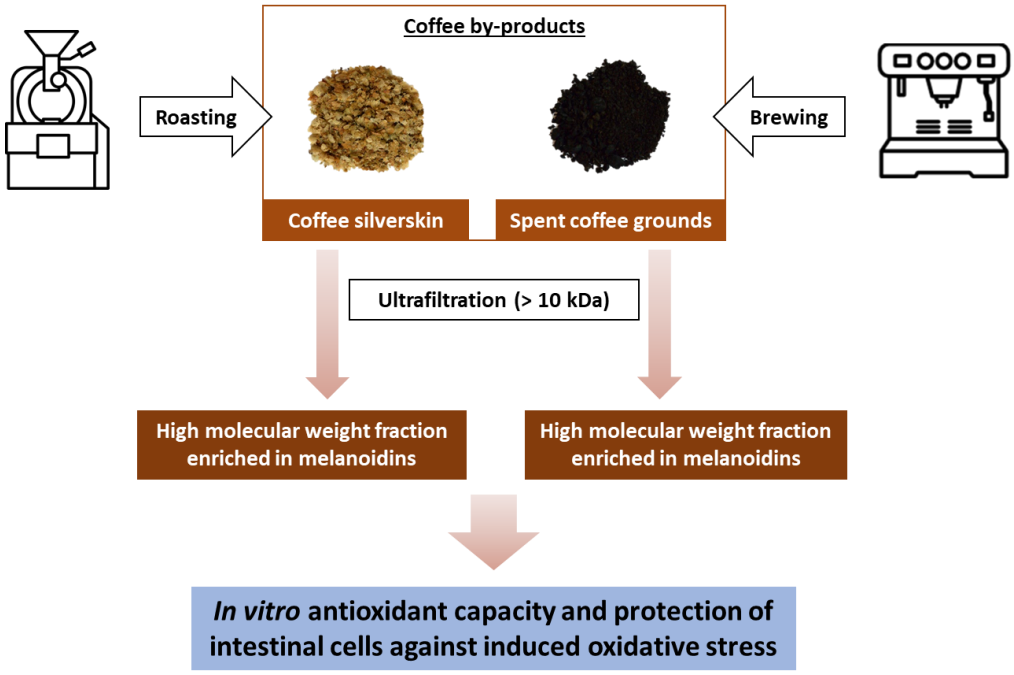
This chapter gives preliminary data on the chemical characterization and assesses the health promoting properties of isolated biomolecules from coffee beans by-products (melanoidins):

- In Study 5, the high molecular weight fraction was isolated from CSE and SCGs by ultrafiltration, and their chemical composition and antioxidant properties on healthy human colon cells were evaluated.
- In Study 6, the structural and functional properties and the bioactivity of melanoidins from CSE were studied *in vivo*.

Study 5: Antioxidant properties of high molecular weight compounds from coffee roasting and brewing byproducts

Results from this chapter have been published in:

Iriondo-DeHond, A., Ramírez, B., & Escobar, F. V. (2019). *Bioactive Compounds in Health and Disease*, 2(3), 48–63. Available at: https://doi.org/10.31989/bchd.v2i3.588



Antioxidant properties of high molecular weight compounds from coffee roasting and brewing byproducts

Amaia Iriondo-DeHond [1], Beatriz Ramirez [1], Francisco Velázquez Escobar [2,3] and Maria Dolores del Castillo [1]*

[1] Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM), C/ Nicolás Cabrera, 9, Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain.

[2] Technische Universität Berlin, 135/PC14 Max Volmer Laboratorium für biophysikalische Chemie, 1023 Berlin, Germany.

[3] Caffeine bioanalytics, Krumme Str. 92, 10585 Berlin, Germany.

Bioactive Compounds in Health and Disease, 2(3), 48–63.

https://doi.org/10.31989/bchd.v2i3.588

Abstract

Coffee is one of the main sources of dietary melanoidins. Coffee melanoidins have antioxidant properties and are associated with protective effects against oxidative damage. The aim of this research was to study the potential of melanoidins obtained from coffee byproducts as functional ingredients to improve gastrointestinal health using normal human colon cells. Melanoidins were extracted from two coffee byproducts: coffee silverskin (CS) and spent coffee grounds (SCGs). Extraction was carried out by ultrafiltration using a 10 kDa molecular cut membrane. Safety of raw materials and isolated fractions was studied by microbiological analysis and determination of acrylamide, respectively. Characterization of coffee isolates was assessed by UV-Vis absorption spectroscopy, infrared spectroscopy, and determination of browning and protein content. The antioxidant capacity was measured by ABTS and formation of intracellular ROS in human intestinal cells (CCD18 cell line). The high molecular weight (HMW) enriched fraction showed antioxidant capacity and protected intestinal cells against induced oxidative stress. Coffee byproducts obtained after the roasting process are a sustainable source of melanoidins that may act as antioxidants and therefore, may have the potential to be used as a functional novel ingredient for the prevention of gastrointestinal diseases caused by oxidative stress.

Keywords: coffee byproducts, melanoidins, gastrointestinal health, sustainable health

1. Introduction

The main sources of dietary melanoidins are coffee and bakery products. Melanoidins are one of the major components of coffee beverages, representing up to the 25 % of dry matter (Belitz *et al.*, 2009). Global dietary intake of melanoidins from coffee ranges from approximately 5 to 40 mg/kg/day (Fogliano & Morales, 2011). Melanoidins comprehend high molecular weight nitrogen-containing compounds that are formed during the roasting process. Here, the green coffee beans experience substantial chemical and structural changes. At a molecular level and as a product of the last stage of the Maillard reaction, polysaccharides, proteins and phenolic compounds are thermochemically transformed into melanoidins (Moreira *et al.*, 2012).

Within the whole coffee fruit value chain, 90 % is discarded as an agricultural waste or byproduct (Iriondo-DeHond *et al*., 2019). Coffee silverskin (CS) and spent coffee grounds (SCGs) are main coffee industry residues (Esquivel & Jiménez, 2012; Mussatto *et al.*, 2011). Since these byproducts are generated after the roasting process of coffee beans, they may be considered an important sustainable source of melanoidins (Mesías & Delgado-Andrade, 2017).

Although the chemical structure of melanoidins is widely unknown, many health-promoting properties have been associated to these compounds, such as antioxidant, antimicrobial, anti-inflammatory, antihypertensive or prebiotic activity (Mesías & Delgado-Andrade, 2017). The most antioxidant melanoidins are those obtained from coffee when compared to other thermally processed foods such as chocolate, sweet wine, balsamic vinegar, beer, bread, breakfast cereals and biscuits (Pastoriza & Rufián-Henares, 2014). The antioxidant capacity of coffee melanoidins has been associated with protective effects against oxidative damage. Studies have shown that the high molecular weight (HMW) fraction of coffee brews subjected to *in vitro* fermentation for 24 h with human fecal bacteria possessed antioxidant activity (Reichardt *et al.*, 2009). Therefore, roasted coffee could be considered as a major source of dietary antioxidants that provide protection to the intestine during a normal gastrointestinal transit time against radical stress in colon.

Considering the vast amount of waste production in current market schemes, it is of great interest to consider coffee byproducts, CS and SCGs as a sustainable source of melanoidins. The aim of the present study was to obtain antioxidant HMW fractions enriched in melanoidins from coffee byproducts implementing a simple, environmental-friendly and sustainable process. Therefore, preliminary studies regarding the potential

effect of risk reduction of gastrointestinal pathologies associated with oxidative stress were carried out in human intestinal cells.

2. Materials and methods

2.1. Raw material

CS from roasted Arabica coffee beans (*Coffea arabica*) was provided by Fortaleza S.A. (Spain). Coffee silverskin extract (CSE) was produced as previously described (del Castillo *et al*., 2013). Briefly, 50 mg of coffee silverskin were added per water milliliter. This mixture was stirred for 10 minutes at 100 ºC and filtered. The filtrate was freeze-dried. Moisture content of powdered CSE was calculated according to AOAC (1999) and it corresponded to 6.5 %.

SCGs from natural 50 % Arabica 50 % Robusta roasted beans from a local restaurant were pelletized for their storage until analysis (PelletSolucion S.L., Salamanca, Spain). Pelletization allowed reducing water content of SCGs. After pelletization, thermal stabilization of SCGs was carried out at 109 °C for 1 hour. Gravimetric measurements were made to constant weight.

2.2. Microbiological analyses of raw materials

CSE and SCGs were microbiologically analyzed to evaluate the safety of their use as food ingredients. Count of (1) total aerobic microorganisms, (2) aerobic microorganisms forming endospores and (3) molds and yeasts were carried out. All assays were performed in sterile conditions, including previous solubilization of 10 g of CSE or SCGs in BPW (90 ml) by using a stomacher (230 rpm, 1 min). Different conditions were set for each analysis: (1) pour plate method, PCA medium, incubation at 30 °C 72 h; (2) pour plate, BHI agar medium, preincubation (80 °C, 10 min) and incubation at 37 °C 48 h; and (3) spread method, SDA with chloramphenicol and incubation at 25 °C 120 h. Results were expressed as colony forming units (CFU)/g.

2.3. Extraction of melanoidins

Prior to ultrafiltration, CSE was prepared in an aqueous solution at 2 mg/ml. Stabilized SCGs were suspended in water at 14 % w/v, incubated at 121 °C for 20 minutes. The soluble fraction was separated by centrifugation at 5,000 rpm for 12 minutes and subjected to gravimetric filtration. A HMW fraction enriched in melanoidins was obtained from CSE and SCGs by ultrafiltration using a 10 kDa Omega 76MM molecular cut membrane with an Amicon Stirred Ultrafiltration Cell. HMW melanoidin fractions

were freeze-dried and stored until analysis. The isolation process was carried out in triplicate.

2.4. Melanoidin fraction characterization

2.4.1. UV-Visible absorption spectroscopy

UV-Vis spectrum of caramel standard and melanoidin enriched fractions (0.5 mg/ml) was acquired using a microplate reader (BioTek Epoch 2 Microplate Spectrophotometer, Winooski, VT, USA). Sulfite ammonia caramel (E-150d) was used as a melanoidin standard. Caramel is formed in a Maillard-type reaction where carbonyl compounds react with amino groups or ammonia (Sengar & Sharma, 2014). Analytical determination was carried out in triplicate. Absorption spectra were recorded between 240 and 720 nm at room temperature.

2.4.2. Infrared spectroscopy (IR)

IR spectra of caramel, CSE, SCGs and corresponding HMW melanoidin fractions were recorded in a Tensor27 (Bruker) mid-infrared spectrometer equipped with a liquid nitrogen cooled MCT-detector, a Rocksolid™ interferometer and a Durascope™ diamond ATR-cell. Prior to sample IR spectrum recording (200 scans), a background reference was measured (400 scans). After each experiment, the diamond crystal was cleaned with distilled water (Millipore-quality) and ethanol (chromatographic grade). IR spectra were analyzed using the OPUS (Bruker) software. Base-line corrected spectra were plotted using the OriginLab Software (V10.X). Characteristic IR bands are observed within a low- (750 - 1800 cm^{-1}) and high-frequency (2700 - 3400 cm^{-1}) window.

2.4.3. Melanoidin content

The content of melanoidins in samples was determined according to that previously described (Silván *et al.,* 2010). HMW extracts were dissolved in water (1 mg/ml) light-absorption at 405 nm was performed using a microplate reader (BioTek Epoch 2 Microplate Spectrophotometer, Winooski, VT, USA). Caramel (E-150d) was used as a melanoidin standard. Analytical determination was carried out in triplicate. Results were expressed in equivalent milligrams of caramel melanoidins/gram of sample.

2.4.4. Soluble proteins

Bio-Rad Protein Assay based on the Bradford method was used in a micro-method format to determine protein concentration. Briefly, a solution of Bradford reagent (1:4, reagent:milli-Q water) was prepared and filtered using Whatman 4 filter. Ten µl of sample and 200 µl of Bradford solution were placed in a multi-well microplate. Samples were incubated for 5 min at room temperature, and the absorbance was measured at 595 nm. Sample blank and reagent blank were also analyzed. A calibration curve was constructed using BSA (0.05 – 0.5 mg/ml). All measurements were performed in triplicate. Results were expressed as mg BSA/g of sample.

2.4.5. Total antioxidant capacity

The trapping capacity of cationic free radicals was evaluated using the method of radical $ABTS^{\bullet+}$ bleaching described for its use in microplate (Oki *et al.*, 2006; Re *et al*., 1999). Aqueous solutions of chlorogenic acid (CGA) were used for calibration (0.15 – 2.0 mM). Absorbance was measured in microplate using a UV–Visible Spectrophotometer (BioTek Epoch 2 Microplate Spectrophotometer, Winooski, VT, USA). All measurements were performed in triplicate and results were expressed as mg CGA equivalents/g of sample.

2.4.6. Acrylamide

Content of acrylamide was determined by liquid chromatography coupled with tandem mass spectrometry with electrospray ionization operated in positive mode (ESI-MS/MS). HWM fractions from CSE and SCGs were dissolved in water (50 mg/ml). Different concentrations of $[^{13}C_3]$-acrylamide (0 – 80 µg/ml) were added as internal standard. Samples (0.85 ml) were treated with 25 µl of each Carrez I and Carrez II solutions and centrifuged 11,600 rpm for 10 min at 4 °C. Supernatants were filtered through 0.45 µm pore diameter nylon membrane syringe filters (Análisis Vínicos, Ciudad Real, Spain). A Varian 1200L, with API-ES between 10 and 1500 Da range mass, liquid chromatograph coupled to a Triple Quadrupole MS detector was used. Acrylamide was recorded using multiple reaction monitoring (MRM) mode by selecting ion m/z 72 $[M^+H]^+$ at the first quadrupole (Q1), fragmented in Q2, and analyzing resulting ion m/z 55 at Q3. The system consisted of two pumps, Varian Prostar 210 and an Phenomenex Aqua C18 (250 mm × 4,6 mm ID, 5 µm,) column, at a flow rate of 0.5 ml/min. Acetonitrile containing 0.1% formic acid (solvent A) and 0.1 % formic acid in H2O (solvent B) as mobile phase was pumped with the following gradient: 10 – 50 % solvent A (0 – 10 min), 50 – 90 % solvent A (10 – 11 min), hold 5 min to recover initial

conditions. The injection volume was 20 µl. The limit of the quantitation was set at 20 µg/kg. Samples and standard solutions were analyzed in triplicate. Acrylamide content was expressed as µg/kg dry weight.

2.5. Cell studies

Normal human colon fibroblasts (CCD-18 cell line) were purchased from the American Type Culture Collection (ATCC, Barcelona, Spain). Cells under passage 10 were cultured as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % v/v heat inactivated fetal calf serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin and 1 % v/v L-glutamine. Cell cultures were incubated at 37 °C and 100 % humidity in a 5 % CO2 atmosphere.

2.5.1. Cytotoxicity

In order to select the non-cytotoxic concentrations for the following intracellular reactive oxygen species (ROS) measurement assays, cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Bakondi *et al.*, 2003). Powdered extracts were prepared in phosphate buffered saline (PBS) solution (10 mM pH 7.4) and sterile filtered. First, CCD-18 cells were cultured at a density of 10^4 cells per well of a 96-well plate for 24 h. Then, cells were treated with caramel or the HMW melanoidin fractions of CSE or SCGs (10 and 25 µg/ml) for 24 h. DMSO (50%) was used as death control. Subsequently, cells were incubated in MTT solution (0.5 mg/ml) for 2 h at 37 ºC. The supernatant was then removed, 100 µl of DMSO were added and the optical density at 570 nm was measured using a microplate reader (BioTek Epoch 2 Microplate Spectrophotometer, Winooski, VT, USA). Experiments were carried out in triplicate. Results were expressed as percentage of viability compared to control non-treated cells.

2.5.2. Intracellular ROS

Intracellular ROS scavenging assay was performed by measuring the fluorescence intensity of the 2',7'-dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe, which is proportional to the amount of ROS formed (Gomes *et al.*, 2005). A 10 mM solution of DCFH-DA was prepared (5 mg in 1 ml DMSO), and a 50 µl aliquot was separated. Then, 750 µl of DMSO were added to the 50 µL solution. CCD-18 cells were cultured at a density of 10^4 cells per well of a 96-well plate for 24 h. After 24 hours of caramel or the HMW melanoidin fractions of CSE or SCGs (10 and 25 µg/ml) incubation, cells were pre-loaded with 2.5 µl/well of DCFH-DA for 30 minutes at 37 ºC. After incubation, DCFH becomes dichlorofluorescein (DCF) due to intracellular oxidants and will emit

fluorescence. Next, culture medium was removed; cells were washed with PBS; and caramel or the HMW melanoidin fractions of CSE or SCGs (10 and 25 µg/ml) were added for 1 hour. Tert-butylhydroperoxide (t-BOOH) 1 mM was used as an oxidation control and vitamin C (10 µg/ml) was used as an antioxidant control. Then, fluorescence was measured at 485 nm/528 nm (BioTek Synergy HT Multi-Mode Microplate Reader). A MTT assay was performed to normalize data by the number of cells per well. Experiments were carried out in triplicate.

To analyze the preventive effect of samples under induced oxidative stress conditions, cells were pretreated with caramel or the HMW fractions of CSE and SCGs in FBS free medium for 24 hours. Then, the experiment was carried out as previously described but cells were treated simultaneously with samples and t-BOOH (1mM) to induce oxidative stress. Experiments were performed in triplicate and results were expressed as % ROS.

2.6. Statistical analysis

Student's T test was carried out for the values obtained from the chemical characterization. One-way analysis of variance (ANOVA) was performed and statistical comparisons of the different treatments were performed using Tukey's test. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using the IBM SPSS Statistics program version 23.

3. Results and discussion

3.1. Microbial safety of raw materials

Food safety is mandatory in order to develop a new food ingredient. However, no microbiological regulations have been stablished for coffee or coffee byproducts. Ochratoxin A (OTA) is one of the several naturally occurring mycotoxins produced by molds that grow on crops or during storage. It is the only mycotoxin subjected to European legislation being the maximum level of 5 µg/kg (European Commission (EC) 1881/2006). With regard to microbial safety of raw CSE, results showed values of 3.25×10^5 CFU/g for endospores, 4.3×10^3 CFU/g of total aerobic microorganisms and a content of yeasts and molds lower than 10^2 CFU/g. Results for raw SCGs showed values below 10^2 CFU/g for endospores, 10^4 CFU/g of total aerobic microorganisms, content of yeasts lower than 10^7 CFU/g, and absence of molds. The absence of molds in our raw materials reduces the risk of OTA contamination. In addition, previous studies have confirmed values below 5 µg/kg for OTA (< 0.3 µg/kg), and other hazardous mycotoxins such as aflatoxin B1 and enniatin B in CSE (Iriondo-DeHond *et al*., 2019). Therefore,

considering microbial safety, CS and SCGs present excellent microbiological quality and may be safe in order to be used as a food ingredient as a source of melanoidins.

3.2. Physico-chemical characterization of HMW fractions

3.2.1. UV-Visible spectral analysis

The UV-Visible spectra of caramel and the HMW fractions of CSE and SCGs in the wavelength regions of 200 - 500 nm are shown in Figure 1. The obtained caramel UV-Vis absorption spectra was similar to that previously reported, in which the HMW caramels absorbed over the full wavelength range of 195 – 600 nm (Royle & Radcliffe, 1999). Measurement of absorption at 280, 325, and 405 nm provides useful information on the relative amount of melanoidins and other compounds in coffee samples (Bekedam *et al.,* 2006). In both HMW coffee byproduct fractions, two absorption maxima were observed at 280 nm and 325 nm, being higher in the HMW-SCGs sample. The absorption maximum at 280 nm in our samples has been also observed in coffee brew samples and it may correspond mostly to the presence of caffeine (Bekedam *et al*., 2006; Belay & Gholap, 2009). The absorption maxima at 280 nm can also be explained by the presence of proteins and phenolic acids. The maximum at 325 nm suggests the presence of chlorogenic and caffeic acid (Belay & Gholap, 2009). There is strong evidence that chlorogenic acid derivatives are components of coffee melanoidins (Moreira *et al.,* 2012). Since chlorogenic acid is a low molecular weight compound (< 10 kDa), the observed maximum at 325 nm might correspond to the chlorogenic acid that forms coffee melanoidins. This absorbance maximum may be due to a contamination of chlorogenic acid that is non-covalently linked to the fiber structure of melanoidins. With additional washing steps after ultrafiltration, such as diafiltration, this contamination could be eliminated. It is generally accepted that melanoidins contain conjugated systems, which result is light absorption throughout the whole spectrum. On the basis of these absorption spectra, it can be stated that absorption measurements at 280, 325, and 405 provide useful information on the relative amounts of melanoidins and other compounds formed (Kang, 2016). Polysaccharides, proteins and chlorogenic acids are involved in coffee melanoidin formation (Moreira *et al*., 2012). The progress of the Maillard reaction involves the production of final and HMW melanoidins with a chromophore group that absorbs around 420 nm (Kang, 2016). Therefore, the suitable range for probing the presence of melanoidins can be delimited to the window between 405 and 420 nm. Here, the absorption of other coffee compounds is rather negligible. Caramel and the HMW fractions obtained from coffee byproducts display a broad

absorption around 405 nm, which is in accordance to the characteristic brown color of melanoidins.

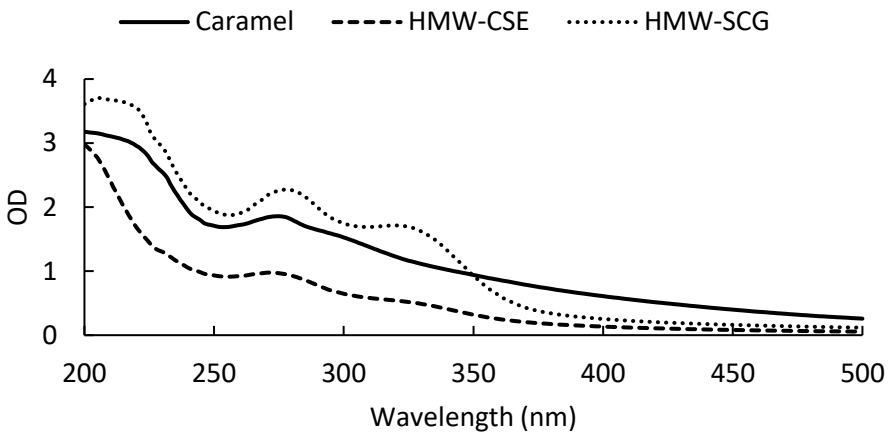


Figure 1. UV-Vis spectra of caramel and the HMW fractions of CSE and SCGs at 0.5 mg/ml.

3.2.2. Infrared spectroscopy (IR)

IR spectroscopy has been widely implemented for the molecular characterization and compound determination of green and roasted coffee beans (Barbin *et al.,* 2014). In addition, IR spectroscopy also provides relevant information regarding bio-compound extraction (Lyman *et al.,* 2003). Here, we report one of the first IR spectra of CSE (Figure 2B), SCGs (Figure 2D) and corresponding HMW fractions (Figure 2C and 2E). The IR spectra of caramel (Figure 2A) was used as a standard reference. For the subsequent vibrational analysis we refer to previous publications (Iriondo-DeHond *et al*., 2019).

Despite a similar brown coloring between caramel (Figure 2A) and the HMW melanoidin fractions, we observe a substantially distinct vibrational pattern. This also refers to the distinct band features of CSE (Figure 2D) and SGG (Figure 2E) melanoidin IR spectra. This suggests an overall specific content of melanoidinic precursors, while the macroscopic color prevails.

With regard to CSE, we observed a broad C-O feature with a maximum at 1022 cm^{-1}, with local maxima at 1044, 1072 and 1096 cm^{-1}. In addition, we observed another prominent at 1601 cm^{-1} with a shoulder at 1645 cm^{-1} and two high-frequency peaks at 1699 and 1734 cm^{-1}. The first peaks corresponded to C=C stretching features whereas the latter ones corresponded to C=O stretching modes. These peaks could be assigned to

the presence of lignin in the sample. Additionally, we observed a broad peak at 1406 cm^{-1} possibly a C-H2/CH3 flowering mode. In the high frequency region, we detected a poorly intense C-H stretching peaks at 2921 and 2851 cm^{-1} and a broad O-H stretching peak at 3311 cm^{-1}. After melanoidin extraction (Figure 2C), we did not observe a sustainable modification of the vibrational features, thus suggesting that the chemical composition prevails.

In contrast, SCGs (Figure 2D) displayed a very strong and broadband envelope with a maximum at 1030 cm^{-1} and local maxima at 1012 and 1055 cm^{-1}, containing mainly contributions of C-O stretching (C-O) coordinates of esters and alcohols. A similar feature is observed in the IR spectra of sugars, cellulose and melanoidins. At 1646 cm^{-1} and 1551 cm^{-1} were assigned to melanoidin-adduct modes or C=C and imidazole stretching modes in caffeine, respectively (in red). These are characteristic peptide bond vibrational features, which, despite partial overlapping with further peaks, could be unambiguously assigned to the protein content in coffee beans (*vide infra*). To higher frequencies, two peaks at 1698 and 1742 cm^{-1} were observed. While both contained C=O stretching coordinates (C=O), the frequency of the first one corresponded to peptide carbonyl C=O modes (amide-I envelope) and the latter one was assigned to the stretching mode of an ester or a protonated carboxylic acid. In this respect, the evidence of two C-H stretching peaks at 2853 and 2921 cm^{-1} and the concomitant disappearance of these features upon extraction (Figure 2E) nicely correlated with the presence of a triglyceric fraction in SCGs, commonly known as roasted coffee oil (green features in Figure 2D). Further peaks at 871 and 3009 cm^{-1} corresponded to this component. In the HMW fraction of SCGs spectrum (Figure 2E), we observed that amide-I and II features as well as the broad peak at 1030 cm^{-1} remained unchanged, whereas the removal of fatty acid content allowed assignment to features at 1596 cm^{-1}, containing C=C stretching character and the rise of less prominent and broad peaks at 1373 and 1449 cm^{-1} (CH_3/CH_2 flowering *vide infra*). In the high frequency region, the broad O-H stretching feature with maxima at 3348 and 3289 cm^{-1} remained unchanged, whereas oil-removal led to the rise of a broad C-H stretching doublet at 2875 and 2929 cm^{-1}. According to previous references, melanoidins had strong and broad O-H stretching peaks and poorly intense C-H features (Rubinsztain *et al.*, 1986).

Both HMW fractions of CSE and SCGs, as well as the CSE displayed characteristic melanoidin-features. In addition, the two distinct samples of each extraction (m1 and m2) displayed almost identical vibrational features (Figure 2C and 2E). The largest discrepancy was observed in the O-H stretching region. While the CSE spectrum mainly

displayed melanoidin-like features, the SCGs spectrum was composed of a melanoidin and a protein component.

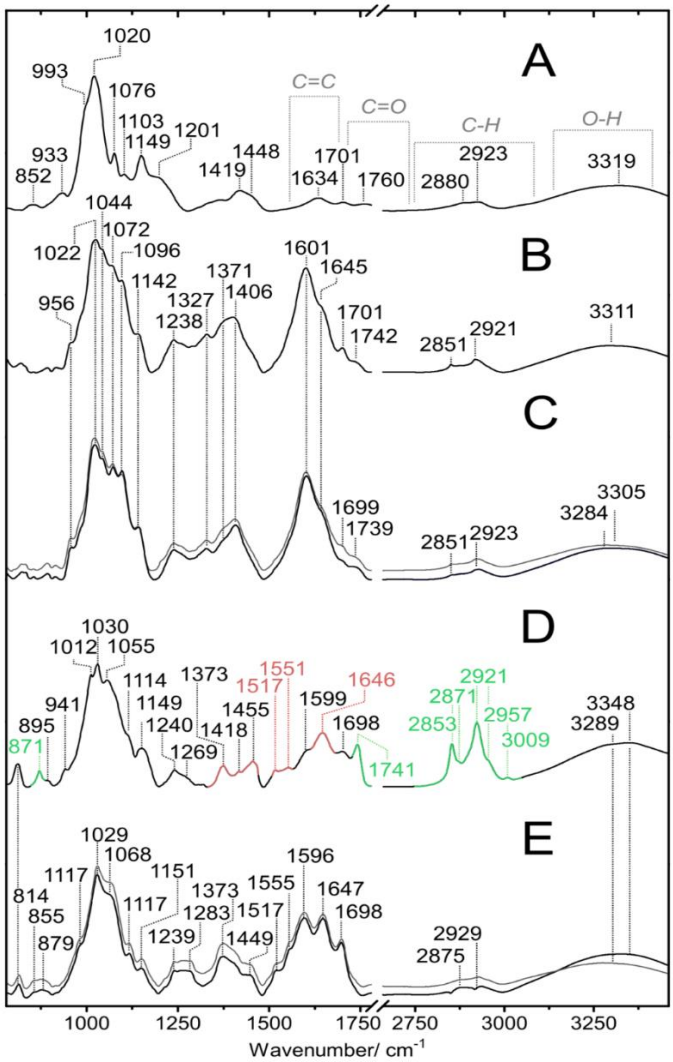


Figure 2. Infrared (IR) spectra of (A) caramel IV dye, (B) coffee silverskin (CS), (C) HMW fraction of coffee silverskin, (D) spent coffee grounds (SCGs) and (E) HMW fraction of spent coffee grounds. For each HMW fraction (C and E) two independent spectra m1 (black) and m2 (grey) were recorded. All relevant band peaks are indicated. Protein (red) and fatty acid (green) contributions are also indicated. Characteristic IR regions with localized potential energy distribution (PED) such as C=C, C=O, C-H and O-H stretching vibration modes are indicated.

3.3. Chemical characterization of the HMW fractions

Table 1 shows the chemical characterization of the HMW fractions of CSE and SCGs. Results showed the presence of melanoidins absorbing at 405 nm in both HMW fractions of coffee byproduct samples. HMW-SCGs showed significantly higher ($p < 0.05$) amount of melanoidins compared to HMW-CSE. Previous studies have reported the amount of melanoidins in these samples, around 4.5 g/100 g for CS and between 13 and 25 g/100 g for SCGs (Borrelli *et al.*, 2004; Martinez-Saez *et al.*, 2017). Coffee melanoidins are diverse and they possess different physicochemical properties. The difference of melanoidin content in raw materials, together with the different species and the different nature of these two byproducts may explain the difference in melanoidin content in the HMW fractions obtained in this study. To the best of our knowledge, this is the first study that has analyzed and compared the HMW fraction of coffee byproducts.

Table 1. Chemical characterization of HMW coffee silverskin extract (CSE) and spent coffee grounds (SCGs) melanoidin fractions.

Analysis	HMW-CSE	HMW-SCGs
Melanoidins (mg eq. caramel/g)	215.79 ± 0.010^{b}	345.96 ± 0.003^{a}
Protein (mg eq. BSA/g)	18.44 ± 1.25^{b}	73.96 ± 3.93^{a}
Total antioxidant capacity (mg eq. CGA/g)	145.72 ± 10.01^{b}	459.93 ± 11.80^{a}
Acrylamide (µg/kg)	< 20	< 20

Data are expressed as the means ± standard deviation (n = 3). Values in each row with different letters differ significantly (T test, $p < 0.05$). BSA, bovine serum albumin; CGA, chlorogenic acid.

Table 1 also shows the total amount of soluble proteins found in HMW-CSE and HMW-SCGs. Previous studies have confirmed the protein-rich composition of these byproducts (Mussatto *et al*., 2011). In this study, protein content in HMW-SCGs was significantly higher ($p < 0.05$) than that observed for HMW-CSE, but values obtained were lower than that found for the original raw materials (Mussatto *et al*., 2011). These results are in accordance to that observed in IR spectra analysis. It is known that the roasting process leads to protein denaturation with degradation and that protein breakdown into small fragments is a major event that takes place during coffee processing (Borrelli *et al.*, 2002; Moreira *et al*., 2012). The different protein profile of green coffee and roasted coffee has been analyzed by SDS-PAGE. Green coffee presented two major protein bands at 58 and 38 kDa. However, roasted coffee presented a defined band at ≤ 14 kDa

and a diffuse band at > 200 kDa. This HMW band > 200 kDa may correspond to proteins forming melanoidins (Nunes & Coimbra, 2001).

The antioxidant capacity of HMW fractions of CSE and SCGs is also shown in Table 1. HMW-SCGs possessed significantly higher ($p < 0.05$) antioxidant capacity compared to HMW-CSE. This difference in antioxidant properties may be due to the significantly higher ($p < 0.05$) content of melanoidins in HMW-SCGs. It is known that coffee melanoidins (HMW fraction of coffee brews) present *in vitro* antioxidant capacity (Borrelli *et al*., 2002). Chlorogenic acids non-covalently linked to the melanoidin skeleton may be important contributors to the overall antioxidant capacity of the HMW fractions of coffee brews (Delgado-Andrade *et al.*, 2005). However, melanoidin fractions without chlorogenic acids have also shown antioxidant capacity, suggesting that low molecular weight polyphenols linked to the central structure of the molecule are not the only contributors of melanoidin's antioxidant capacity (Gniechwitz *et al*., 2008). Different results have been published on the effect of roasting on the antioxidant capacity of the melanoidin fractions isolated from coffee brews. With regard to results obtained from $ABTS^{+}$ assay, some authors have shown higher antioxidant capacity in lighter degrees of roast (Borrelli *et al*., 2002). In contrast, other authors showed that the antioxidant activity of melanoidins isolated by ultra-filtration from instant coffees was lower in the light-roasted samples (Delgado-Andrade *et al*., 2005). Further research regarding the study of melanoidin's structure is needed in order to better understand their antioxidant properties.

The presence of Maillard reaction products derived from lysine such as N^{ε}-(fructosyl)lysine (FL), N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL), have been identified in the HMW fraction of coffee brews (Nunes & Coimbra, 2007). Therefore, the content of another product from the Maillard reaction derived from asparagine, acrylamide, was analyzed. Human population is widely exposed to acrylamide. This compound has been classified as a Group 2A carcinogen (probably carcinogenic to humans) by the International Agency for Research on Cancer (IARC) due to its neurotoxic, genotoxic, carcinogenic and reproductive toxicity properties (European Food Safety Authority, 2015). As expected, levels < 20 μg/kg of acrylamide were found in HMW samples since this compound has low molecular weight (71.08 Da) (Table 2). Acrylamide content in samples was much lower than that established by the European Commission for roasted coffee (400 μg/kg) and instant coffee (850 μg/kg) (European Commission, 2017). The insignificant amounts found of acrylamide proved the food safety of our samples. Levels of 489 and 37 μg/kg of acrylamide have been previously found in CSE and SCGs, respectively (Iriondo-DeHond *et al*., 2019;

Martinez-Saez *et al.*, 2017). Since acrylamide is a water-soluble compound, it is normally transferred to the beverage during the brewing process (Stadler & Theurillat, 2012). This may explain the lower content of acrylamide in SCGs compared to CSE.

3.4. Cell studies

Previous to the evaluation of the effect of HMW fraction of coffee byproducts on intracellular ROS, the effect of samples on cell viability was studied. Table 2 shows the viability of human fibroblasts isolated from normal colon tissue (CCD-18 cells) after 24 hours of incubation with HMW-CSE and HMW-SCGs. Concentrations of 10 and 25 μg/ml were selected after previous preliminary studies. Caramel was again used as a melanoidin standard. None of the tested concentrations of HMW-CSE, HMW-SCGs and caramel (10 μg/ml) resulted cytotoxic for CCD-18 cells ($p > 0.05$). No previous studies have reported the effect of melanoidins from coffee byproducts in healthy human colon fibroblasts.

Obtained results regarding the effect of the HMW fraction of CSE and SCGs on the production of intracellular ROS on CCD-18 cells is shown in Figure 3. Cells treated with t-BOOH (oxidation control) presented significantly higher ($p < 0.05$) levels of intracellular ROS. Vitamin C (10 μg/ml) was included as an antioxidant standard and significantly reduced ($p < 0.05$) values of intracellular ROS. Caramel and samples significantly reduced ($p < 0.05$) levels of physiological intracellular ROS compared to non-treated cells (100 % ROS) (Figure 3A). No significant differences ($p > 0.05$) in the reduction of physiological intracellular ROS were observed between samples.

Once the harmless effect of the HMW fraction of coffee byproducts on cell viability and physiological intracellular ROS was confirmed, the potential protective effect against induced oxidative stress was studied (Figure 3B). A significant increase ($p < 0.05$) in ROS production was observed when cells were treated with t-BOOH 1 mM for 1 hour compared to non-stressed controls. Vitamin C (10 μg/ml) used as an antioxidant control also prevented the formation of induced intracellular ROS. Pretreatment of CCD-18 cells with caramel (10 μg/ml) and HMW-CSE and HMW-SCGs (10 or 25 μg/ml) for 24 hours prevented ROS production induced by t-BOOH, since a significant decrease ($p < 0.05$) in ROS levels was observed. Similar results regarding the absence of cytotoxicity and the effect of coffee melanoidins in the redox status of cells has been previously observed (Goya *et al.*, 2007). However, melanoidin samples used by these authors were obtained from coffee brews and were studied on liver cancer cells. In this study, melanoidins were obtained from a sustainable source since coffee byproducts are wastes generated by the

coffee industry that are produced worldwide in large amounts and cause environmental problems.

Table 2. Effect of 24 h treatment with noted concentrations of HMW fractions of coffee silverskin extract (CSE) or spent coffee grounds (SCGs) in CCD-18 cells determined by the MTT assay. Control, non-treated cells. DMSO (50%) was used as a death control.

Treatment	**Cell viability (%)**
Control	100.0 ± 8.5 [a]
Death control	6.0 ± 1.1 [c]
Caramel	
10 µg/ml	99.7 ± 21.3 [a]
25 µg/ml	70.9 ± 13.9 [b]
HMW-CSE	
10 µg/ml	109.0 ± 20.2 [a]
25 µg/ml	86.9 ± 10.7 [a]
HMW-SCGs	
10 µg/ml	91.6 ± 19.2 [a]
25 µg/ml	89.4 ± 18.0 [a]

Data represent means ± SD of 3 independent experiments. Different letters denote statistically significant differences between treatments (Tuckey test, $p < 0.05$).

Normal intestinal CCD-18 cells were used in this research. Although no previous studies have been published regarding the effect of coffee melanoidins in normal intestinal cells, other authors have studied melanoidins from different sources in different cell lines. For instance, Martín *et al*. (2009) have studied the effect of similar concentrations of biscuit melanoidins (0.5 – 10 µg/ml) in HepG2 liver cells. Authors observed a significant dose-response effect ($p < 0.05$) in the reduction of physiological intracellular ROS also using the DCFH-DA probe (Martín *et al*., 2009). However, when HepG2 cells were under oxidative stress conditions, biscuit melanoidins < 10 kDa had no effect in the reduction of induced intracellular ROS. In contrast, when cells were pre-treated with intermediate mass biscuit melanoidins (3 – 10 kDa), a significant decrease ($p < 0.05$) in induced intracellular ROS was observed (Martín *et al*., 2009). When digested coffee melanoidins were studied also in HepG2 cells using the same approach, increased ROS generation induced by t-BOOH was not prevented (Goya *et al*., 2007). In addition, melanoidins from thermally treated apricots protected endothelial cells, related to the development of

cardiovascular diseases, from hydrogen peroxide-induced intracellular oxidation, mitochondrial depolarization and cell death (Cossu *et al*., 2012).

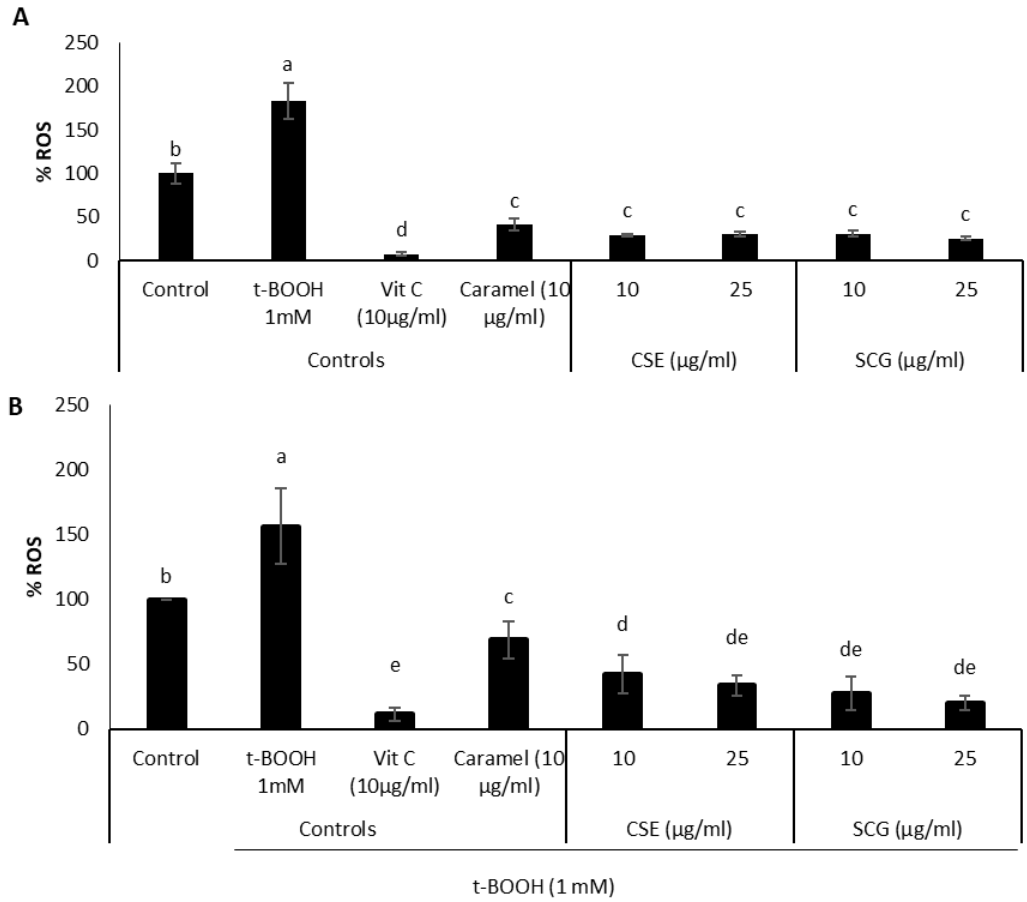


Figure 3. Effect of treatment with noted concentrations of caramel and HMW-CSE and HMW-SCGs on physiological (A) or induced (B) intracellular ROS in CCD-18 cells determined by the DCFH-DA probe. t-BOOH (1 mM) was used as oxidative damage control. Cells were pre-treated with samples or Vit C (10 µg/ml) for 24 h and incubated with the DCFH-DA probe for 30 minutes. Then, cells were treated with samples in absence (A) or presence (B) of t-BOOH 1 mM. Finally, fluorescence of intracellular ROS was measured, and data represent means ± SD of 4 independent experiments. Different letters denote statistically significant differences between all treatments (Tukey test, $p < 0.05$).

During coffee roasting melanoidins develop a more complex structure, which comprehends non-covalent incorporation of low molecular weight compounds such as chlorogenic acids to the initial backbone constituted by carbohydrates, including dietary

fiber, polyphenols and proteins (Mesías & Delgado-Andrade, 2017). Therefore, melanoidins may be a type of "Maillardized dietary fiber" acting as dietary fiber with an overall antioxidant capacity leverage by embedded low molecular compounds (Silván *et al.,* 2010). Results obtained in this study indicate that melanoidins extracted from CSE and SCGs possess *in vitro* antioxidant capacity and show protective effects against induced oxidative stress in intestinal cells. Consequently, melanoidins from coffee byproducts have great potential to be used as sustainable novel food ingredients to prevent or reduce the risk of gastrointestinal pathologies associated with oxidative stress. Further studies are required, especially to unveil a more accurate structural insight of these specific melanoidins' type. This detailed picture will substantially contribute to a comprehensive understanding of their potential health-promoting implications.

4. Conclusions

Two extracts enriched in melanoidins were obtained from coffee silverskin and spent coffee grounds. Both extracts have shown antioxidant properties *in vitro* and in normal human intestinal cells. Results suggest the use of the obtained sustainable antioxidant melanoidins as a novel functional ingredient with potential for reducing the risk of gastrointestinal pathologies associated with oxidative stress.

Acknowledgments and Funding: The SUSCOFFEE Project (AGL2014-57239-R): Sustainable coffee production and consumption: Valorization of coffee waste into food ingredients funded this work. A. Iriondo-DeHond is a fellow of the FPI predoctoral program of MINECO (BES-2015-072191). F.VE. acknowledges the support of Prof. Dr. Peter Hildebrandt (TU Berlin). Authors thank the Servicio Interdepartamental de Investigación (SIdI UAM) for the analysis of acrylamide in samples.)

Author Contributions: Methodology and Data Curation, A.I., B.R: and F.V.E.; Writing Original Draft Preparation and Writing-Review & Editing, A.I., F.V.E. and M.D.C.; Supervision, Project Administration, Funding Acquisition and Writing-Review & Editing, M.D.C.

Competing Interests: There are no conflicts of interest to declare.

References

Bakondi, E., Gönczi, M., Szabó, É., Bai, P., Pacher, P., Gergely, P., … Virág, L. (2003). Role of intracellular calcium mobilization and cell-density-dependent signaling in oxidative-stress-induced cytotoxicity in HaCaT keratinocytes. *Journal of Investigative Dermatology*, *121*(1), 88–95. https://doi.org/10.1046/j.1523-1747.2003.12329.x.

Barbin, D. F., Felicio, A. L. de S. M., Sun, D. W., Nixdorf, S. L., & Hirooka, E. Y. (2014). Application of infrared spectral techniques on quality and compositional attributes of

coffee: An overview. *Food Research International, 61*, 23–32. https://doi.org/10.1016/j.foodres.2014.01.005.

Bekedam, E. K., Schols, H. A., van Boekel, M. A. J. S., & Smit, G. (2006). High Molecular Weight Melanoidins from Coffee Brew. *Journal of Agricultural and Food Chemistry*, *54*(20), 7658–7666. https://doi.org/10.1021/jf0615449.

Belay, A., & Gholap, A. (2009). Characterization and determination of chlorogenic acids (CGA) in coffee beans by UV-Vis spectroscopy. *African Journal of Pure and Applied Chemistry*, *3*(11), 234–240. https://doi.org/10.5897/AJPAC.

Belitz, H.-D., Grosch, W., & Schieberle, P. (2009). Coffee, tea, cocoa. In: *Food Chemistry*. H.D. Belitz, W. Grosch and P. Schieberle, (Eds.), 4th edition, Springer, Leipzig (pp. 938–951).

Borrelli, R. C., Esposito, F., Napolitano, A., Ritieni, A., & Fogliano, V. (2004). Characterization of a new potential functional ingredient: coffee silverskin. *Journal of Agricultural and Food Chemistry*, *52*(5), 1338–1343. https://doi.org/10.1021/jf034974x.

Borrelli, R. C., Visconti, A., Mennella, C., Anese, M., & Fogliano, V. (2002). Chemical Characterization and Antioxidant Properties of Coffee Melanoidins. *Journal of Agricultural and Food Chemistry*, *50*(22), 6527–6533. https://doi.org/10.1021/jf025686o.

Cossu, A., Posadino, A. M., Giordo, R., Emanueli, C., Sanguinetti, A. M., Piscopo, A., … Pintus, G. (2012). Apricot Melanoidins Prevent Oxidative Endothelial Cell Death by Counteracting Mitochondrial Oxidation and Membrane Depolarization. *PLoS ONE*, *7*(11). https://doi.org/10.1371/journal.pone.0048817.

del Castillo, M. D., Ibañez, M. E., Amigo, M., Herrero, M., Plaza del Moral, M., & Ullate, M. (2013, January). *Application of products of coffee silverskin in anti-ageing cosmetics and functional food*. WO 2013/004873.

Delgado-Andrade, C., Rufián-Henares, J. A., & Morales, F. J. (2005). Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. *Journal of Agricultural and Food Chemistry*, *53*(20), 7832–7836. https://doi.org/10.1021/jf0512353

Esquivel, P., & Jiménez, V. M. (2012). Functional properties of coffee and coffee by-products. *Food Research International*, *46*(2), 488–495. https://doi.org/10.1016/j.foodres.2011.05.028.

European Commission. (2017). COMMISSION REGULATION (EU) 2017/2158 of 20 November 2017 establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food. *Official Journal of the European Union*,

204, 24–44. https://doi.org/http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_285/l_28520031101en00330037.pdf.

European Food Safety Authority. (2015). Scientific Opinion on acrylamide in food. *EFSA Journal*, *13*(6), 4104. https://doi.org/10.2903/j.efsa.2015.4104.

Fogliano, V., & Morales, F. J. (2011). Estimation of dietary intake of melanoidins from coffee and bread. *Food & Function*, *2*(2), 117–123. https://doi.org/10.1039/c0fo00156b.

Gniechwitz, D., Reichardt, N., Meiss, E., Ralph, J., Steinhart, H., Blaut, M., & Bunzel, M. (2008). Characterization and fermentability of an ethanol soluble high molecular weight coffee fraction. *Journal of Agricultural and Food Chemistry*, *56*(14), 5960–5969. https://doi.org/10.1021/jf800231q.

Gomes, A., Fernandes, E., & Lima, J. L. F. C. (2005). Fluorescence probes used for detection of reactive oxygen species. *Journal of Biochemical and Biophysical Methods*, *65*(2–3), 45–80. https://doi.org/10.1016/j.jbbm.2005.10.003.

Goya, L., Delgado-Andrade, C., Rufián-Henares, J. a, Bravo, L., & Morales, F. J. (2007). Effect of coffee melanoidin on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. *Molecular Nutrition & Food Research*, *51*(5), 536–545. https://doi.org/10.1002/mnfr.200600228.

Iriondo-DeHond, A., Aparicio García, N., Velazquez Escobar, F., San Andres, M. I., Sanchez-Fortun, S., Blanch, G. P., … del Castillo, M. D. (2019). Validation of coffee by-products as novel food ingredients. *Innovative Food Science and Emerging Technologies*, *51*, 194–204. https://doi.org/10.1016/j.ifset.2018.06.010.

Kang, O. J. (2016). Evaluation of melanoidins formed from black garlic after different thermal processing steps. *Preventive Nutrition and Food Science*, *21*(4), 398–405. https://doi.org/10.3746/pnf.2016.21.4.398.

Lyman, D. J., Benck, R., Dell, S., Merle, S., & Murray-Wijelath, J. (2003). FTIR-ATR analysis of brewed coffee: Effect of roasting conditions. *Journal of Agricultural and Food Chemistry*, *51*(11), 3268–3272. https://doi.org/10.1021/jf0209793.

Martín, M. Á., Ramos, S., Mateos, R., Rufián-Henares, J. A., Morales, F. J., Bravo, L., & Goya, L. (2009). Biscuit melanoidins of different molecular masses protect human HepG2 cells against oxidative stress. *Journal of Agricultural and Food Chemistry*, *57*(16), 7250–7258. https://doi.org/10.1021/jf9006032.

Martinez-Saez, N., Tamargo García, A., Domínguez Pérez, I., Rebollo-Hernanz, M., Mesías, M., Morales, F. J., … del Castillo, M. D. (2017). Use of spent coffee grounds as food ingredient in bakery products. *Food Chemistry*, *216*, 114–122. https://doi.org/10.1016/j.foodchem.2016.07.173.

Mesías, M., & Delgado-Andrade, C. (2017). Melanoidins as a potential functional food ingredient. *Current Opinion in Food Science*, *14*, 37–42. https://doi.org/10.1016/j.cofs.2017.01.007.

Moreira, A. S. P., Nunes, F. M., Domingues, M. R., & Coimbra, M. A. (2012a). Coffee melanoidins: structures, mechanisms of formation and potential health impacts. *Food & Function*, *3*(9), 903–915. https://doi.org/10.1039/c2fo30048f.

Mussatto, S. I., Machado, E. M. S., Martins, S., & Teixeira, J. a. (2011). Production, Composition, and Application of Coffee and Its Industrial Residues. *Food and Bioprocess Technology*, *4*(5), 661–672. https://doi.org/10.1007/s11947-011-0565-z.

Nunes, F. M., & Coimbra, M. A. (2001). Chemical characterization of the high molecular weight material extracted with hot water from green and roasted arabica coffee. *Journal of Agricultural and Food Chemistry*, *49*(4), 1773–1782. https://doi.org/10.1021/jf0012953.

Nunes, F. M., & Coimbra, M. A. (2007). Melanoidins from coffee infusions. Fractionation, chemical characterization, and effect of the degree of roast. *Journal of Agricultural and Food Chemistry*, *55*(10), 3967–3977. https://doi.org/10.1021/jf063735h.

Oki, T., Nagai, S., Yoshinaga, M., Nishiba, Y., & Suda, I. (2006). Contribution of b-Carotene to Radical Scavenging Capacity Varies among Orange-fleshed Sweet Potato Cultivars. *Food Science and Technology Research*, *12*, 156–160.

Pastoriza, S., & Rufián-Henares, J. A. (2014). Contribution of melanoidins to the antioxidant capacity of the Spanish diet. *Food Chemistry*, *164*, 438–445. https://doi.org/10.1016/j.foodchem.2014.04.118.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3.

Reichardt, N., Gniechwitz, D., Steinhart, H., Bunzel, M., & Blaut, M. (2009). Characterization of high molecular weight coffee fractions and their fermentation by human intestinal microbiota. *Molecular Nutrition and Food Research*, *53*(2), 287–299. https://doi.org/10.1002/mnfr.200700509.

Royle, L., & Radcliffe, C. M. (1999). Analysis of caramels by capillary electrophoresis and ultrafiltration. *Journal of the Science of Food and Agriculture*, *79*(12), 1709–1714. https://doi.org/10.1002/(SICI)1097-0010(199909)79:12<1709::AID-JSFA425>3.0.CO;2-X.

Rubinsztain, Y., Yariv, S., Ioselis, P., Aizenshtat, Z., & Ikan, R. (1986). Characterization of melanoidins by IR spectroscopy-I. Galactose-glycine melanoidins. *Organic Geochemistry*, *9*(3), 117–125. https://doi.org/10.1016/0146-6380(86)90101-4.

Sengar, G., & Sharma, H. K. (2014). Food caramels: a review. *Journal of Food Science and Technology*, *51*(9), 1686–1696. https://doi.org/10.1007/s13197-012-0633-z.

Silván, J. M., Morales, F. J., & Saura-Calixto, F. (2010). Conceptual study on maillardized dietary fiber in coffee. *Journal of Agricultural and Food Chemistry*, *58*(23), 12244–12249. https://doi.org/10.1021/jf102489u.

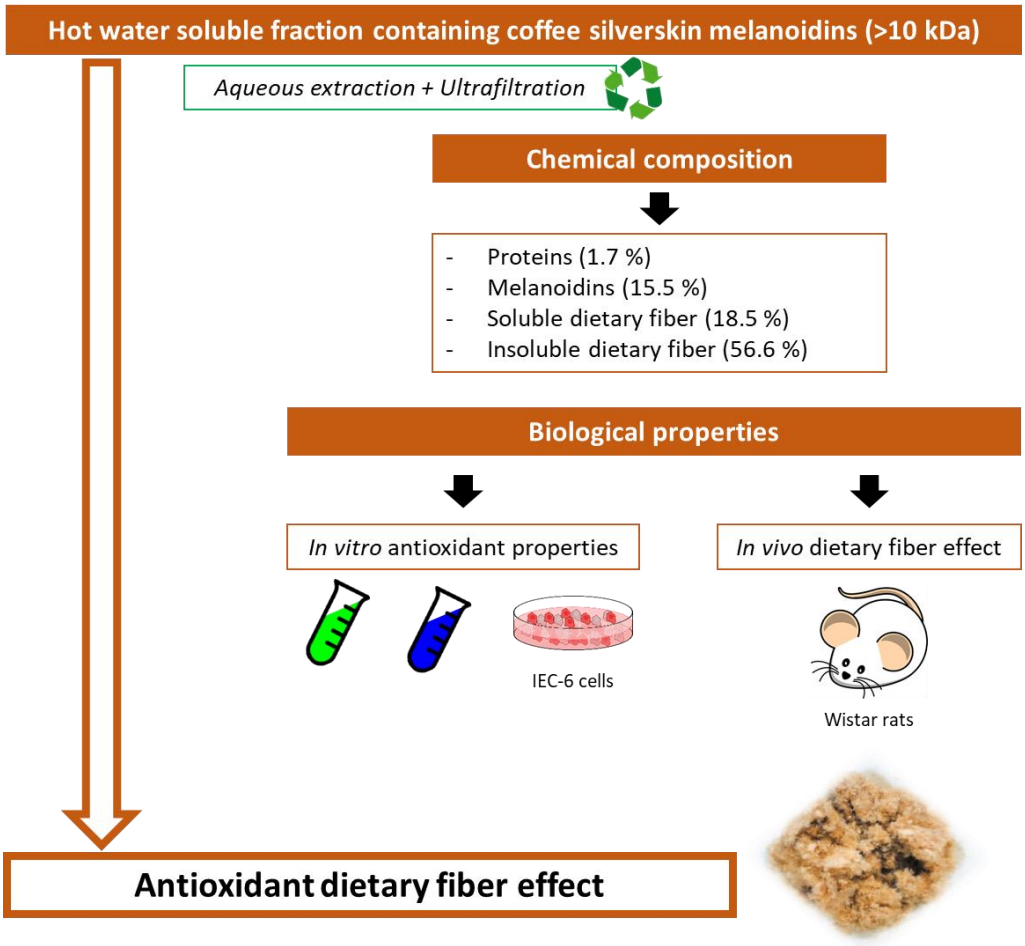
Silván, J. M., Morales, F. J., & Saura-Calixto, F. (2010). Conceptual Study on Maillardized Dietary Fiber in Coffee. *Journal of Agricultural and Food Chemistry, 58*(23), 12244–12249. https://doi.org/10.1021/jf102489u.

Stadler, R. H., & Theurillat, V. (2012). Acrylamide in Coffee. In *Coffee: Emerging Health Effects and Disease Prevention*, Y-F. Chu (Ed.), 259–273. Wiley-Blackwell https://doi.org/10.1002/9781119949893.ch15.

Study 6: An assessment of the bioactivity of coffee silverskin melanoidins

Results from this chapter have been published in:

Tores de la Cruz, S., Iriondo-DeHond, A., Herrera, T., Lopez-Tofiño, Y., Galvez-Robleño, C., Prodanov, M., Velazquez-Escobar, F., Abalo, R., Castillo, M. D. del. (2019). *Foods*, 8(2), 68. Available at: https://doi.org/10.3390/foods8020068



An Assessment of the Bioactivity of Coffee Silverskin Melanoidins

Silvia Tores de la Cruz [1], Amaia Iriondo-DeHond [1], Teresa Herrera [1], Yolanda Lopez-Tofiño [2], Carlos Galvez-Robleño [2], Marin Prodanov [1], Francisco Velazquez-Escobar [3], Raquel Abalo [2] and Maria Dolores del Castillo [1]*

[1] Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM), Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain.

[2] Departamento de Ciencias Básicas de la Salud, Facultad de Ciencias de la Salud, Universidad Rey Juan Carlos (URJC), Alcorcón, 28922 Madrid, Spain.

[3] Technische Universität Berlin, 135/PC14 Max Volmer Laboratorium für biophysikalische Chemie, 1023 Berlin, Germany.

Foods, 2019, 8(2), 68.

https://doi.org/10.3390/foods8020068

Abstract

Melanoidins present in coffee silverskin, the only by-product of the roasting process, are formed via the Maillard reaction. The exact structure, biological properties, and mechanism of action of coffee silverskin melanoidins, remain unknown. This research work aimed to contribute to this novel knowledge. To achieve this goal, melanoidins were obtained from an aqueous extract of Arabica coffee silverskin (WO2013004873A1) and was isolated through ultrafiltration (>10 kDa). The isolation protocol was optimized and the chemical composition of the high molecular weight fraction (>10 kDa) was evaluated, by analyzing the content of protein, caffeine, chlorogenic acid, and the total dietary fiber. In addition, the structural analysis was performed by infrared spectroscopy. Antioxidant properties were studied in vitro and the fiber effect was studied in vivo, in healthy male Wistar rats. Melanoidins were administered to animals in the drinking water at a dose of 1 g/kg. At the fourth week of treatment, gastrointestinal motility was evaluated through non-invasive radiographic means. In conclusion, the isolation process was effective in obtaining a high molecular weight fraction, composed mainly of dietary fiber, including melanoidins, with *in vitro* antioxidant capacity and *in vivo* dietary fiber effects.

Keywords: antioxidant; coffee byproduct; dietary fiber; gastrointestinal motility; melanoidins; Maillard reaction; silverskin

1. Introduction

Melanoidins are widely distributed in foods and are part of our daily diet. These compounds are found in coffee, bakery products, boiled potatoes, cocoa, toasted barley, beer, caramel, and sweet wine. Coffee and bakery products are the most important sources of melanoidins. It is estimated that the daily intake of melanoidins from these sources is approximately between 1.5 and 6 g for the average consumer (Marta Mesías & Delgado-Andrade, 2017). Melanoidins are high molecular weight brown polymeric compounds generated during the last stage of the Maillard reaction (Marta Mesías & Delgado-Andrade, 2017). Melanoidins are responsible for color, taste and texture of foods submitted to high temperatures (Pérez-Hernández *et al*., 2011). There are two main types of melanoidin structures present in foods - melanoidins of polysaccharide type, such as those described in coffee brew; and protein-based melanoidins, predominant in bakery products (Marta Mesías & Delgado-Andrade, 2017). Extraction of melanoidins can be carried out by different techniques (Echavarría *et al*., 2012). Dialysis and diafiltration with membranes from the ultrafiltration scale are the most frequently used procedures for elimination of low molecular weight compounds and recovery of an enriched fraction of high molecular weight compounds such as polysaccharides, proteins and melanoidins (Nunes & Coimbra, 2010). The most used technique for the isolation of coffee melanoidins is ultrafiltration, with 10 kDa molecular weight cut-off membranes. Ultrafiltration presents various advantages, such as treatment of huge volume extracts for short time, no phase change, no solvents use, complete physical separation and easy industrial scaling.

Coffee silverskin may be an important source of melanoidins. Coffee silverskin is the tegument of the outer layer of the coffee bean, representing approximately 4.2 % (w/w) of the coffee cherry and is the only byproduct produced during coffee roasting (Iriondo-DeHond *et al*., 2019). The roasting of four tons of coffee produces about 30 kg of coffee silverskin (Alves *et al*., 2017). During the roasting process, melanoidins are transformed into a more complex structure, where low molecular weight compounds, such as chlorogenic acids, bind non-covalently to the initial skeleton, constituted by carbohydrates, dietary fiber, polyphenols and proteins (Marta Mesías & Delgado-Andrade, 2017). The total dietary fiber in aqueous coffee silverskin extract (CSE) has been reported to be about 36% (Guglielmetti *et al*., 2019).

Coffee melanoidins possess various biological properties such as anticarcinogenic, anticariogenic, antiglycative, antihypertensive, anti-inflammatory, antimicrobial, antioxidant, and prebiotic (Marta Mesías & Delgado-Andrade, 2017; Pérez-Hernández

et al., 2011; Wang *et al.*, 2011). Melanoidins from coffee have been described as "Maillardized dietary fiber" (Silván *et al.*, 2010). Coffee silverskin has been proposed as a sustainable natural source of prebiotics, antioxidants and dietary fiber (del Castillo *et al.*, 2013). Borrelli *et al.* (2004) described for the first time that coffee silverskin supports the growth of bifidobacteria *in vitro*, and subsequently Jiménez-Zamora *et al.* (2015) have evaluated the prebiotic properties of different coffee byproducts (Jiménez-Zamora *et al.*, 2015). Coffee silverskin significantly increased the amount of healthy bacteria, such as *Lactobacillus spp*. and *Bifidobacterium spp*. without affecting the level of *Bacteroides spp*. and *Clostridium spp*. The authors concluded that coffee silverskin could be a suitable ingredient in the formulation of foods with prebiotic characteristics. The antioxidant capacity of coffee silverskin extract is due to melanoidins generated during the roasting process (Esquivel & Jiménez, 2012), and also to the presence of chlorogenic acid (CGA) (Murthy *et al.*, 2009). Determining the contribution of each of these compounds to the overall antioxidant capacity of the extract is of great interest. Additionally, the dietary fiber effect of melanoidins from coffee silverskin has not been investigated yet. Therefore, the aim of the present study was to isolate and perform a structural and functional characterization of melanoidins obtained from coffee silverskin, and to analyze the antioxidant properties *in vitro* and the dietary fiber effect *in vivo*.

2. Materials and methods

2.1. Raw material

Coffee silverskin Arabica species from Colombia was kindly provided by Supracafé S.A. (Móstoles, Spain). Coffee silverskin was generated during the roasting process of green coffee beans in a PROBAT roaster (Emmerich am Rhein, Germany) for 13 min at 220 °C.

2.2. Preparation of coffee silverskin extract

Coffee silverskin extract (CSE) was prepared according to the procedure described in patent WO2013004873A1 (del Castillo *et al.*, 2013). Briefly, a mixture of 50 g/L of coffee silverskin, in water, was stirred for 10 min at 100 °C, then the suspension was screened by 250 µm and freeze-dried. The extraction yield of CSE was 10.09 %, which corresponds to that previously described (Iriondo-DeHond *et al.*, 2019).

2.3. Recovery of melanoidin-rich macromolecular fraction

A simple, easy to scale up, cost-friendly and sustainable process, avoiding the use of potential harmful solvents for human health, was performed to obtain water soluble melanoidins. Molecules with this physicochemical property are of great interest because of their physiological effect as a soluble dietary fiber. In addition, water solubility facilitates their administration in drinkable water, for the animal model, avoiding stress caused by the use of gavage, in the gastrointestinal tract.

The filtrate obtained after water extraction of coffee silverskin was clarified by dead-end filtration, using cellulose pads (SA-590) from Filtrox Southern Europe S.L. (Besós, Spain) and vacuum as driving force. The melanoidin fraction (120.32 g) was obtained by aqueous extraction from 3.76 kg of coffee silverskin in 75.2 L of hot water, corresponding to a concentration of 50 g CS/L.

Melanoidins (MEL) were recovered as the macromolecular fraction of coffee silverskin, through ultrafiltration, using a 10 kDa nominal molecular weight cut-off membrane from Millipore (Merck, Darmstadt, Germany) (model, Prep-scale 6; material, regenerated cellulose; design, spiral-wound; filtration surface, 0.54 m^2). The membrane was integrated in a tangential (cross) flow, pressure-driven membrane filtration unit, consisting of a 5 L feed vessel, variable flow peristaltic pump (1-13 L/min), membrane holder with integrated inlet and outlet pressure gauges (1-5 bar). Ultrafiltration was carried out in a continuous concentration mode, at a constant transmembrane pressure of 0.8 bar and temperature of 22 °C until 1.5 L of concentrate were achieved. Recovery of the macromolecular (colloidal) fraction was carried out by diafiltration of the final concentrate with the same volume of demineralized water (batch mode), until 0.1 % of total dissolved substances (TDS) (°Brix) were registered in the permeate flow. This effect was achieved within six cycles of dilution/concentration, equivalent to 9 L of water per 1.5 L of concentrate. The purified fraction was freeze-dried and stored at room temperature in a dark place until analyses.

After filtration, the membrane was cleaned by 0.1 N NaOH solution, according to producer´s requirements.

Both ultrafiltration and diafiltration stages were monitored by spectrophotometric measurement of caffeine (CAF) at 273 nm and melanoidins at 405 nm (BioTek Instruments, United States), and measurement of pH and turbidity.

2.4. Microbiological quality

The melanoidin beverage (4 g/L) prepared form the purified melanoidin-rich macromolecular fraction of coffee silverskin was microbiologically analyzed to evaluate the safety of its use as a food ingredient. A count of the (i) total aerobic microorganisms, (ii) aerobic microorganisms forming endospores, and (iii) molds and yeasts, was carried out. All assays were performed in sterile conditions, with a previous solubilization of the melanoidin fraction (10 g) in buffered peptone water (BPW) (90 ml), by using a stomacher (230 rpm, 1 min). Different conditions were set for each analysis: (i) the pour plate method, plate count agar (PCA) medium, incubation at 30 °C, for 72 h; (ii) the pour plate method, brain heart infusion (BHI) agar medium, pre-incubation at 80 °C, for 10 min and incubation at 37 °C, for 48 h; and (iii) the spread method, sabouraud dextrose agar (SDA) medium with chloramphenicol and incubation at 25 °C, for 120 h. Results were expressed as colony forming units (CFU)/g.

2.5. Spectral analyses

2.5.1. Ultraviolet-visible (UV-VIS) absorption spectroscopy

UV-VIS spectrum of caramel reference substance, CSE, MEL, CGA (0.06 mg/ml) and CAF (0.03 mg/ml) were acquired, using a microplate reader (BioTek Epoch 2 Microplate Spectrophotometer, Winooski, VT, USA). Sulphite ammonia caramel (E-150d) was used as a melanoidin standard. Caramel is formed during Maillard-type reaction where carbonyl compounds react with amino groups or ammonia (Sengar & Sharma, 2014). Therefore, it was used as a melanoidin control. Analytical determination was carried out in triplicate. Absorption spectra were recorded between 240 and 720 nm at room temperature. Monitorization of the isolation process was carried out by the analysis of the UV-VIS absorption spectra.

2.5.2. Infrared spectroscopy (IR)

The infrared spectra of caramel standard, CSE and MEL were recorded in a Tensor27 FT-spectrometer (Bruker, Billerica, USA), equipped with a diamond attenuated total reflection (ATR) cell (Durascope, Smiths Detection, Danbury, CT, USA). A background reference was measured (400 scans) previous to sample infrared spectrum recording (200 scans). After each experiment, the diamond crystal was cleaned with distilled water (Millipore quality) and ethanol (chromatographic grade). The infrared spectra were analyzed using the OPUS software (Bruker). The corrected spectra of the baseline were plotted, using the OriginLab software (V10.X, OriginLab Corporation, Northampton,

MA, USA). Characteristic infrared red bands are observed within a low window (750-1800 cm^{-1}) and high frequency (2700-3400 cm^{-1}).

2.6. Chemical analyses

2.6.1. Caffeine and chlorogenic acid

The content of CAF and CGA was determined using capillary zone electrophoresis (CZE) as described by del Castillo *et al*., (2002), in an attempt to identify the bioactive compounds present in samples. Determinations were carried out by an Agilent G1600 A (Santa Clara, CA, USA) capillary electrophoresis instrument equipped with ChemStation software and a diode array detector (DAD). The capillary was 48.5 cm long (40 cm to the detector) with an internal diameter of 50 µm and a x3 bubble cell. Other conditions of analysis were as follows: 20 mM borate buffer at pH 9.3; voltage, 20 kV; temperature of analysis set at 25 °C; injection administered at 50 mbar for 5 s; and an electroosmotic flow (EOF) marker of acetone. Electrophoregrams (e-grams) were monitored at 280 nm, and spectra were collected from 190 to 600 nm. The capillary was conditioned after each sample run by flushing with 0.1 M NaOH, for 3 min, and with a buffer for other 3 min. CAF and CGA were used as standards for identification and quantification. All analyses were performed in triplicate, and results were expressed as % CAF or CGA (w/w).

2.6.2. Melanoidins

The content of melanoidins was determined by CZE, as previously described. Caramel (E-150d) was used as reference. The analyses were performed in triplicate, and results were expressed as % (w/w) of caramel equivalents.

2.6.3. Soluble proteins

Bio-Rad Protein Assay, catalogue number 500-006, (Bio-Rad Laboratories, SIG 093094) based on the method of Bradford was used in micro-method format to determine protein concentration. It was performed according to the manufacturer's instructions. Briefly, a solution of Bradford reagent (1:4, reagent:milli-Q water) was prepared and filtered using Whatman 4 filter. Ten µl of sample and 200 µL of Bradford solution were placed in a multi-well microplate. Samples were incubated for 5 min at room temperature, and the absorbance was measured at 595 nm. Sample blank and reagent blank were also analyzed. A calibration curve was constructed using BSA (0.05–0.5 mg/ml). All measurements were performed in triplicate. Results were expressed as % (w/w).

2.6.4. Dietary fiber

Insoluble (IDF), soluble (SDF) and total (TDF) dietary fiber content was determined using the Total Dietary Fiber Assay Kit (Megazyme International Ireland, Ireland) according to manufacturer's instructions, and based on the enzymatic–gravimetric method. All measurements were performed in triplicate. Results are expressed as percent *(%).*

2.7. Bioactivity evaluation

2.7.1. *In vitro*

2.7.1.1. ABTS Assay

The trapping capacity of cationic free radicals was evaluated using the method of radical $ABTS^{\bullet+}$ bleaching described by Re *et al*., (1999) and modified by Oki *et al.,* (2006) for its use in a microplate. Aqueous solutions of Trolox (0.025 – 0.2 mM) and CGA (0.025 – 0.2 mM) were used for calibration. Absorbance was measured at 734 nm using a UV-Visible Spectrophotometer (BioTek Instruments, USA). All measurements were performed in triplicate and results were expressed as µmol Trolox equivalents (TE)/ mg sample.

2.7.1.2. ORAC Assay

The ORAC assay was applied according to the method of Ou *et al*. (2001), and as modified by Dávalos *et al*. (2005). The procedure was carried out using an automated plate reader (BMG LABTECH, Germany) equipped with a fluorescence detector set at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Readings were taken every minute, for a duration of 90 min, at 37 °C. All measurements were performed in triplicate and results were expressed as µmol TE/ mg sample.

2.7.1.3. Intracellular reactive oxygen species (ROS)

Normal rat small intestine epithelial cells (IEC-6) were kindly provided by the Bioanalytical Techniques Unit (BAT) of the Instituto de Investigación en Ciencias de la Alimentación (CIAL) (Madrid, Spain). Cells were cultured as a monolayer in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % (v/v) heat inactivated fetal calf serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin and 1 % (v/v) L-glutamine, at 37 °C and in 5 % CO_2 in a humidified incubator (BINDER CB series 2010, Tuttlingen, Germany).

Prior to the study of basal intracellular ROS, the effect of different concentrations of CSE and melanoidins on cell viability was measured by the MTT assay (Bakondi *et al*., 2003) in order to select non-cytotoxic doses. IEC-6 cells were treated with samples at 0.004, 0.04, 0.4 and 4 mg/ml. DMSO (50%) was used as death control.

The determination of basal intracellular ROS was performed, following the same procedure used by Iriondo-DeHond *et al*. (2019). Tert-butyl hydroperoxide (tBOOH) 1 mM was used as a positive oxidation control and vitamin C (10 µg/ml) was used as an antioxidant reference compound. Induced intracellular ROS were also measured, when combining samples and tBOOH 1 mM. Then, a MTT assay was performed to normalize data by the number of cells per well. Experiments were carried out in triplicate.

2.7.2. *In vivo*

2.7.2.1. Animals and experimental design

Male Wistar rats (150 - 200 g) were obtained from the Veterinary Unit at Universidad Rey Juan Carlos (URJC) (n = 14). Animals were group-housed (2 - 4 rats/cage) in standard transparent cages (60 cm × 40 cm × 20 cm), under environmentally controlled conditions (temperature = 20°C; humidity = 60%), with a 12-hour light/12-hour dark cycle. Animals had free access to standard laboratory rat chow (Harlan Laboratories Inc., Barcelona, Spain) and sterile tap water. All experimental procedures were approved by the Ethics Committee of URJC and carried out in accordance with the EU Directive for the protection of animals used for scientific purposes (2010/63/EU) and Spanish regulations (RD/53/2013).

Animals were divided in two groups, control group (n = 8) and melanoidins group (n = 6). Although the MEL beverage is within the established limits for molds and aerobes when compared to tea, it was sterilized at 121 °C for 20 minutes, and was stored at 4 °C. A fresh MEL bottle was prepared daily to administer to the animals. MEL were administered in drinking water at 4 g/l corresponding to a dose of 1 g/kg of body weight (Oecd/Ocde, 1995). A pilot study validated the oral route for administration of melanoidins in the drinking water. Exposure to normal water or water with melanoidins was performed during four consecutive weeks. Throughout this time, general parameters were regularly evaluated (body weight, water and food intake, and appearance of animals). In the last week, specific parameters of gastrointestinal and colonic motility were also evaluated.

To assess possible dehydration, the dorsal skin fold was evaluated (Hickman & Swan, 2010). In addition, the appearance of the perianal area as well as the fecal pellets were

evaluated (Fukudome *et al*., 2014). Each parameter was evaluated separately. This assessment was made every day.

2.7.2.2. Radiographic study of gastrointestinal motility

The analysis of gastrointestinal motility was carried at the end of the fourth week of the study out by radiographic, non-invasive, *in vivo* methods routinely used by the Pharmacology Laboratory of the URJC (Abalo *et al*., 2009; Cabezos *et al*., 2008). Radiographs were taken 0, 1, 2, 4, 6, 8 h (T0 - T8) after administration of barium contrast (Barigraf® AD; Juste SAQF, Madrid, Spain) at 3 ml/rat (Barigraf was suspended in distilled water at 2 g/ml). Radiographs were developed using an automatic processor (Kodak X-OMAT 2000).

For the analysis of the radiographs, after their digitalization, a semiquantitative scale was applied to each gastrointestinal region (the stomach, small intestine, caecum and the colorectal region) of each rat and at each time point, obtaining scores between 0 and 12, which were represented in the corresponding motility curves (Cabezos *et al*., 2008). In addition, the alterations in the size and density of stomach, caecum and fecal pellets were analyzed with the aid of an image analysis system (Image J 1.38 for Windows, National Institute of Health, USA, free software: https://rsb.info.nih.gov/ij/).

2.7.2.3. *In vivo* evaluation of colonic bead expulsion

Colonic bead expulsion test was performed, as described previously (Fichna *et al*., 2014). Briefly, on the day of the experiment, a prewarmed (37°C) glass bead (diameter: 8 mm) with fire polished end was inserted 3 cm into the distal colon using a silicone pusher (Broccardo *et al*., 2004). Before insertion, beads were covered with vaseline to avoid tissue damage. After bead insertion, rats were separated into transparent, individual cages and the time to bead expulsion was measured (Fichna *et al.*, 2009). Animals were monitored for a maximum of 4 h unless the bead expulsion occurred sooner. Bead insertion was performed under sedation with Sedator® (medetomidine hydrochloride, an alpha-2 adrenergic agonist, 0.66 ml/kg, 5 mg/ml, ip); immediately after bead insertion, Revertor® (atipamezole hydrochloride, an alpha-2 adrenergic antagonist, 0.66 ml/kg, 5 mg/ml, ip) was used to revert sedation (Ruíz *et al*., 2018).

2.7.2.4. Extraction of organs at sacrifice

At the end of the study, animals were guillotined under anesthesia with sodium pentobarbital (2 ml/kg), the gastrointestinal package was extracted for evaluation of the length of the small intestine and colon, and samples were obtained for further studies.

2.8. Statistical Analysis

GraphPad Prism program version 5.01 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. Results are shown as means ± standard deviation (SD) or ± standard error of mean (SEM). Differences between groups were analyzed using Student's T test for unpaired data. A one- or two-way analysis of variance (ANOVA) followed by Bonferroni (gastrointestinal motility analysis) or Tukey's (intracellular ROS analysis) test for mean comparisons was used to highlight significant differences among samples. Differences were considered to be significant at $p < 0.05$ and highly significant at $p < 0.01$.

3. Results and discussion

3.1. Coffee silverskin melanoidins recovery

For recovery of the melanoidin-rich macromolecular fraction, five batches of 12 l of clarified crude coffee silverskin extract were submitted to ultrafiltration, with the equipment, and at the operating conditions already described in the Materials and methods section. Concentration was carried out at 0.8 bar of transmembrane pressure, for an average mean time of 2 h. Even the initial extract had relatively low content of total dissolved (0.6 - 0.8 g/100 ml) and suspended solids (20 - 25 Nephelometric Turbidity Units (NTU)); the filtration flux declined quickly from 18 to 8 l/hm^2. Addition of demineralized water during the diafiltration process did not improve filtration, maintaining the flux at levels between 8 and 2 l/hm^2. This finding suggests that the gel layer build on the membrane surface was quite stable. That is why another 3 to 4 h were necessary to completely separate the low molecular weight species from the macromolecular fraction.

Measurement of total soluble substances (TSS) of the permeate and concentrate streams at the beginning and the end of the concentration phase showed that the 10 kDa membrane retained from 50 % to 70 % of the extract soluble matter, which corresponded to the retained macromolecular fraction. Diafiltration of this retentate, with demineralized water and freeze-drying of the low-molecular-weight-free macromolecular fraction, allowed the recovery of 25 g solid dry matter per batch. The extraction yield of the MEL fraction was 3.2 %. In this way, 126 g of purified melanoidin-rich fraction were obtained for the *in vivo* experimental study.

TSS increased progressively until reached 2 °Brix, pH values were constant, ranging between 5.0 - 5.28, turbidity increased continuously, and the content of dissolved salts decreased progressively. No bibliographical references have been found regarding pH

values of CSE, although the pH values for coffee beverages ranged from 4.9 - 5.6 (Paiva *et al*., 2018). Hashimoto *et al*. (2011) described that acidity is a positive quality of coffee beverage and is attributed to CGA, citric, tartaric and malic acids. Compounds that contribute to a higher or lower pH and acidity are classified as dissociable and non-dissociable ionic species; within the dissociable ionic compounds are CGA, citric acid, quinic acid and trigonelline, whereas the non-dissociable ionic compounds include caffeine and arabinogalactan (Hashimoto *et al.*, 2011).

The MEL fraction was analyzed microbiologically to evaluate the safety of its use as a food ingredient. The permitted values for molds in teas are 10^4 CFU/g of the product and, for mesophilic aerobic colonies (30 °C) the permitted values are up to 10^6 CFU/g of the product; being our results below these established limits. With regard to the microbiological safety of coffee, only the maximum permitted level for ochratoxin A (OTA) is legislated. Previous studies carried out by our research group showed values of 4.3 x 10^3 CFU/g for total aerobic microorganisms and a content of yeasts and molds lower than 10^2 CFU/g in CSE (Iriondo-DeHond *et al.*, 2017), and OTA was not detected (Iriondo-DeHond *et al*., 2019). Table 1 shows the microbiological analyses of the MEL beverage, according to current legislation for tea and its derivatives, regulated by RD 1354/83, BOE 05/27/83 and BOE 14/07/84.

Table 1. Microbiological analyses of the melanoidin beverage (MEL) prior to its administration to rats.

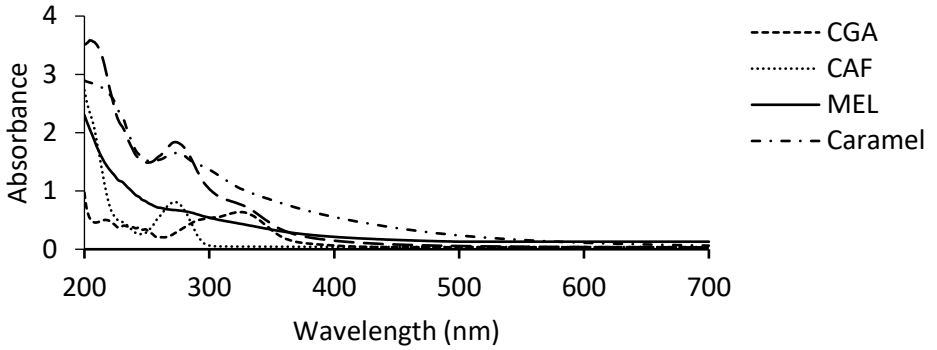
Microbiological Analyses	**MEL**
Molds (CFU/g)	$< 10^2$
Yeasts (CFU/g)	4.8 x 10^4
Total aerobic microorganisms (CFU/g)	4.8 x 10^4
Endospores 30 °C (CFU/g)	$< 10^1$
Viable aerobic microorganisms at 30 °C (CFU/g)	9.4 x 10^3

Figure 1a shows the UV-Visible spectrum of CAF, caramel, CGA, CSE and MEL. CAF spectrum shows a maximum absorbance at 273 nm and the CGA spectrum shows a peak at 326 nm. In CSE, these two peaks are also observed. The maximum absorption at 273 nm may be due to the presence of proteins, CAF and caffeic acid and the maximum absorption at 326 nm might correspond to the presence of CGA (Bekedam *et al*., 2008). Both CAF and CGA are low molecular weight nonpolar compounds (Bekedam *et al.*, 2008). Furthermore, it was observed that CSE absorbs at 400 - 450 nm, which may be due to the presence of melanoidins, previously described to absorb in this wavelength

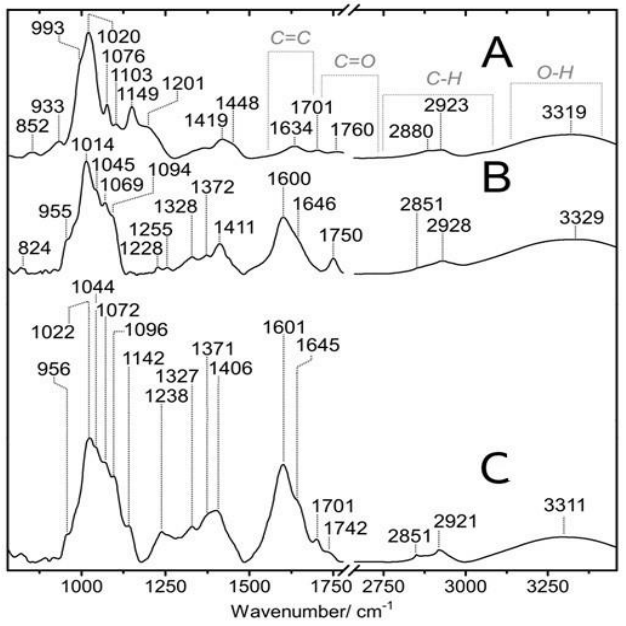
interval (Silván *et al*., 2010). MEL fraction obtained after diafiltration showed absorption in the whole spectrum (270 - 450 nm), which might indicate the presence of melanoidins and traces of caffeine. Caramel (E-150d) has a similar spectrum to that observed in MEL. Melanoidins are soluble anionic polymeric compounds that are brown in color. These compounds have a molecular weight higher than 10 kDa, and absorb in the interval between 400 and 450 nm (Silván *et al*., 2010).

As an extended approach to standard analytical characterization, a spectral analysis in CSE and MEL was performed using vibrational spectroscopy as a non-invasive technique in the middle infrared region. This technique has been widely used for the molecular characterization and determination of green and roasted coffee beans compounds (Barbin *et al*., 2014). In addition, infrared spectroscopy also provides relevant information on the extraction of biocomposites (López-Lorente & Mizaikoff, 2016). Little is known about the implementation of infrared spectroscopy in the structural characterization of coffee by-products and the sustainable management of food, such as coffee silverskin. Figure 1b shows one of the first high-quality IR spectra of MEL (Figure 1(b)B), compared with the IR spectrum of CSE (Figure 1(b)C) and with the IR spectrum of caramel standard (Figure 1(b)A). Despite a similar brown coloration between caramel and MEL, a substantially different vibratory pattern is observed. In the caramel spectrum a poor or pseudo-melanoidinic band is found, which suggests that all the melanoidin extracts related to coffee show an equal mixture of reducing sugars and amino acids. In CSE a wide characteristic of C - O is observed around 1030 cm^{-1}, but the maximum was displaced to 1022 cm^{-1}, with local maximums of 1044, 1072 and 1096 cm^{-1}. In addition, another prominent maximum is observed at 1601 cm^{-1} with one shoulder at 1645 cm^{-1} and two high-frequency peaks at 1699 and 1734 cm^{-1}. The first peaks correspond to C = C stretch characteristics, while the latter corresponded to stretch modes C = O. In addition, a broad peak is observed at 1406 cm^{-1}, possibly to a flowering mode C-H_2 / CH_3. In the high frequency region, little intense CH stretching peaks were detected at 2921 and 2851 cm^{-1} and a wide peak of OH stretching was seen at 3311 cm^{-1}. After the extraction, no changes in the vibratory characteristics were observed, which suggests that the composition of the chemical prevailed. Both CSE and MEL showed melanoidin characteristics. In addition, two different samples of each extraction showed almost identical characteristics. The structure of the coffee parchment, a by-product of the coffee bean, was analyzed by infrared spectroscopy and it was concluded that the components present were lignin and cellulose. The bands at 1658 and 1602 cm^{-1} were assigned to lignin. In high frequency region the peaks at 2896 and 2712 cm^{-1} were assigned to cellulose (Iriondo-DeHond *et al*., 2019). Taking into account that the dietary

fiber of coffee has been described to be composed mainly of cellulose, hemicellulose, pectic substances and lignin (Borrelli *et al.*, 2004) and presents values close to those found for the parchment, it could be suggested that lignin is present in its structure.



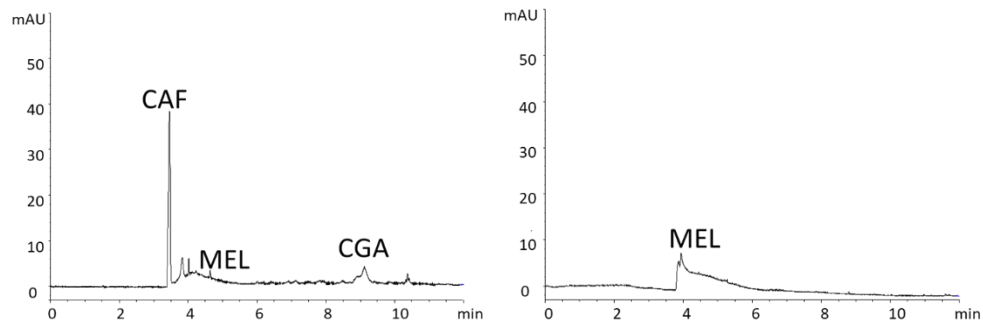
(a)



(b)

Figure 1. (a) UV-VIS spectra of caramel, melanoidins (MEL), coffee silverskin extract (CSE), caffeine (CAF) and chlorogenic acid (CGA). (b) IR spectra of caramel (A), coffee silverskin extract (B) and melanoidins (C). Relevant bands and vibrational modes are indicated.

CAF and CGA were determined by CZE. Identified compounds are shown in Figure 2. The e-gram of CSE showed two peaks, with a spectrum matching CAF and CGA, migrating at 3.4 min and 9.1 min, respectively. For comparative purposes, the identified compounds of CSE were quantified by CZE (Table 2). CAF and CGA content in coffee CSE were 34.87 ± 1.85 mg/g extract and 11.50 ± 0.98 mg/g extract, respectively. In addition, a broad band is observed after 4 to 8 min, similar to that of coffee drinks and representing the colored macromolecules formed in the Maillard reaction, that is, melanoidins, as is described in the literature (Charurin *et al.*, 2002). These spectral characteristics of the melanoidins confirm their presence in CSE. Figure 2b shows the MEL isolate and, as expected, the same broad band is observed after 4 min, as in CSE, its maximum being found at 4.8 min.



(a) (b)

Figure 2. E-gram recorded at 280 nm showing the identified compounds from coffee silverskin (4 mg/ml) (a) and melanoidins (4 mg/ml) (b). Peak identification: CAF - caffeine; CGA - chlorogenic acid; MEL - melanoidins.

Table 2 shows the chemical composition of CSE and MEL and their antioxidant capacity. Results indicate that melanoidins, proteins, CGA and CAF are found in CSE. The amount of melanoidins present in CSE corresponds to 7.8 g/100 g, similar to that previously described (Ii *et al.*, 2004). However, no bibliographical references have been found regarding the isolation and characterization of coffee silverskin melanoidins. The caffeine content corresponds to that previously described by Mesías *et al*., (2014). The European Food Safety Authority (EFSA) has established a level of safety for daily caffeine consumption of 400 mg for the general population and 200 mg for lactating women (European Food Safety Authority (EFSA), 2015). Previous studies have indicated that the caffeine content in coffee by-products are not worrisome (Garcia-Serna *et al.*, 2014). The results obtained for CGA are lower than those found in other

studies (Ii *et al.*, 2004). The antioxidant capacity of CSE is partially due to the presence of compounds generated during the Maillard reaction, melanoidins (Ii *et al*., 2004).

Table 2. Chemical composition and *in vitro* bioactivity of coffee silverskin extract (CSE) and melanoidins (MEL).

Measure (% w/w)*	**CSE**	**MEL**
Caffeine	3.5 ± 0.2^{a}	0.1 ± 0.0^{b}
Chlorogenic acid	1.2 ± 0.1^{a}	0.2 ± 0.0^{b}
Melanoidins	7.8 ± 0.2^{a}	14.6 ± 0.8^{b}
Protein	2.2 ± 0.0^{a}	1.7 ± 0.0^{a}
Dietary fiber	N.D	75.1 ± 4.8
Total antioxidant capacity (µmol Trolox/ mg sample)		
ABTS	4.0 ± 1.2^{a}	1.8 ± 0.1^{b}
ORAC	4.4 ± 0.2^{a}	1.1 ± 0.0^{b}

*Weight per freeze-dried sample. N.D.: not determined. Data represent mean ± SD of 3 independent experiments. Different letters represent statistically significant differences (Student's t-test, $p < 0.05$).

MEL presented low levels of CGA, CAF and proteins, and high melanoidin content (Table 2). The low values of protein (1.7 g/100 g) suggest that MEL is mainly composed of polysaccharides. In previous investigations, the high molecular weight fraction was isolated from the coffee beverage (Arabica variety) and the main compounds found were polysaccharides (37 g/100 g), followed by proteins (12 g/100 g) (Moreira *et al.*, 2017).

The main compound present in the MEL fraction obtained in this study was TDF (75.1 %). Recommendations regarding the consumption of TDF vary depending on the different regulations. For instance, the World Health Organization (WHO) recommends a daily intake of 27 to 40 g of TDF, the Food and Drug Administration (FDA) recommends the consumption of 25 g of TDF per day while the American Dietetic Association (ADA) recommends consuming between 20 and 30 g/day. Among TDF content, IDF and SDF were 56.6 % and 18.5 %, respectively.

According to the literature, the CSE has a TDF value of 62.4 g/100 g, the IDF content being higher (53.7 g/100 g) than the SDF content (8.8 g/100 g) (Pourfarzad *et al*., 2013). No studies in the literature were found to have looked into the chemical characterization of the MEL fraction from the roasting by-product of coffee processing. Low molecular weight compounds, such as CGA, adhere to the structure of melanoidins during roasting,

leading to an antioxidant dietary fiber. Coffee silverskin fiber has been previously classified as an antioxidant dietary fiber (Murthy & Naidu, 2012; Napolitano *et al*., 2007). SDF and IDF have different physiological functions, largely depending on the viscosity of fibers. In the large intestine, they promote a regulatory effect: IDF (found in cellulose, hemicellulose, lignin and resistant starch) mechanically excites the intestinal mucosa, by stimulating it and cause secretion of water; and SDF (found in inulin, pectins, gums and fructooligosaccharides) forms a gel with high water retention capacity. To exert its regulatory effect, dietary laxative fibers must resist fermentation, and remain relatively intact and present throughout the large intestine, consequently increase the water content in feces (McRorie & McKeown, 2017). In this study, an isolate enriched extract in Maillardized dietary fiber has been obtained from coffee silverskin. Taking into account the physiological properties of SDF and IDF in gastrointestinal health, it is of great interest to evaluate the bioactivity of this sustainable source of fiber *in vitro* and *in vivo*.

3.2. Bioactivity of coffee silverskin melanoidins

3.2.1. Antioxidant capacity

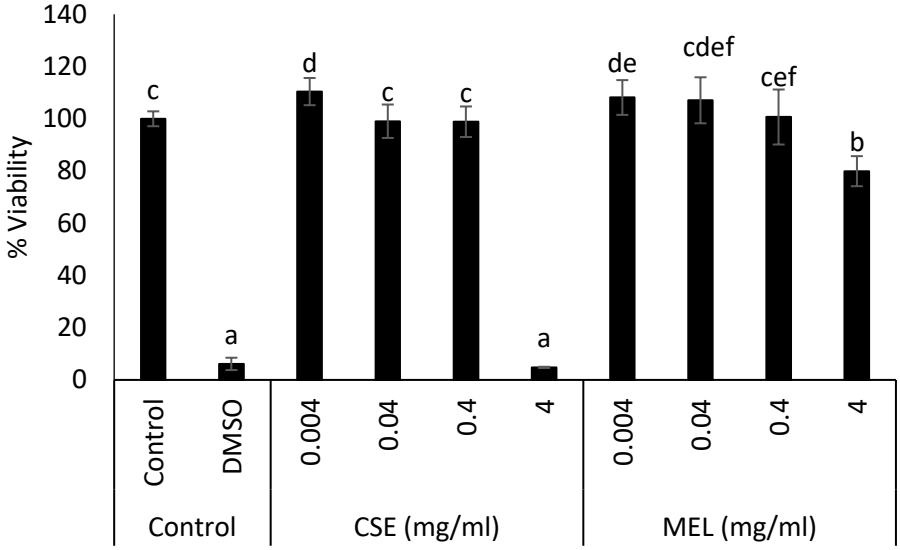
The *in vitro* antioxidant capacity of CSE and MEL, determined by ABTS and ORAC, is shown in Table 2. Antioxidant capacity was significantly higher ($p < 0.05$) in CSE compared to MEL. This trend was observed for both methods. Results regarding the antioxidant capacity of CSE agree with those described in the literature. The antioxidant capacity of CSE may be due to the phenolic compounds present in the raw material and to the presence of melanoidins formed during roasting (Borrelli *et al*., 2004). It is suggested that these low molecular weight phenolic compounds, such as CGA, are covalently bound to the carbohydrate backbone, forming a fiber-antioxidant complex (Hofmann & Schieberle, 2002). Since the MEL fraction obtained in this study was diafiltrated, non-bound low molecular weight compounds were eliminated and, therefore, it was expected to have a lower antioxidant capacity. Even so, results indicated that MEL has antioxidant properties and contributes about 35 % to the total antioxidant capacity of CSE. No bibliographical references have been found regarding the total antioxidant capacity of MEL from coffee silverskin.

Prior to the evaluation of the effect of MEL on intracellular ROS formation, cell viability assays were performed to select non-cytotoxic concentrations of extracts. Figure 3a shows the viability of healthy rat intestinal cells (IEC-6) after 24 h treatment with CSE and MEL, at different concentrations. The highest concentration of CSE and MEL (4 mg/ml) significantly reduced ($p < 0.05$) cell viability, when compared to non-treated

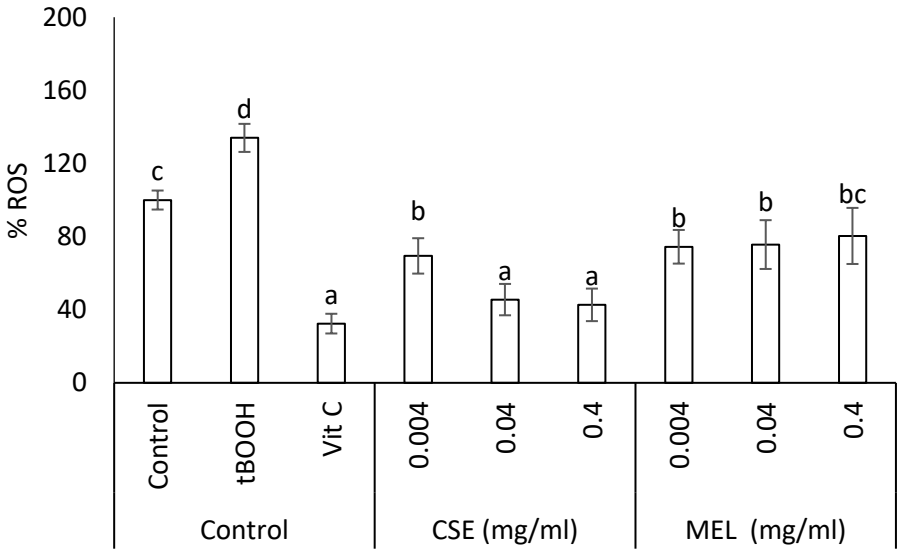
control cells. Therefore, lower concentrations of samples (0.004, 0.04 and 0.4 mg/ml) that resulted non-cytotoxic to intestinal cells were selected for the study of their effect on the redox status of cells.

Figure 3b shows the effect of CSE and MEL on the physiological ROS of IEC-6 cells after 24 h of incubation. Both extracts significantly reduced ($p < 0.05$) the physiological production of ROS compared to the control (untreated cells). Concentrations of 0.04 and 0.4 mg/ml of CSE had similar antioxidant effect ($p > 0.05$) as vitamin C (10 µg/ml) used as an antioxidant control.

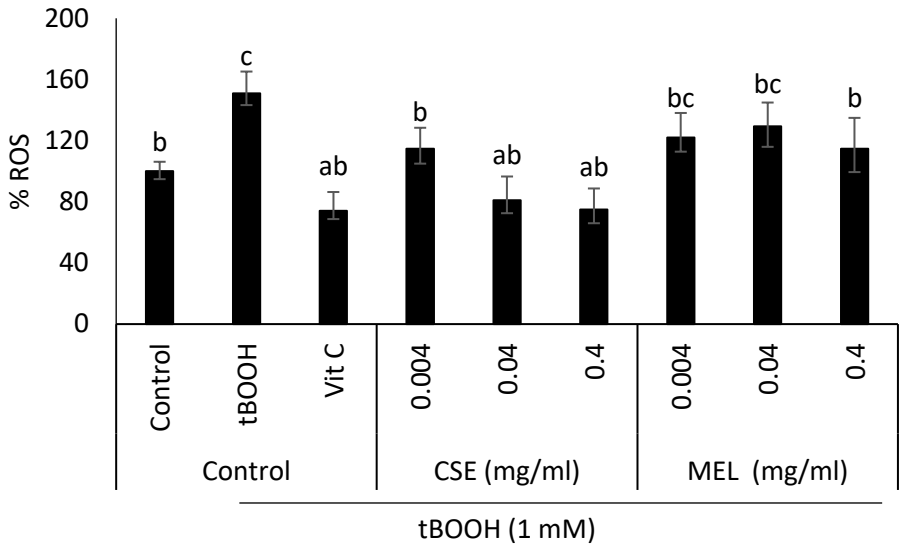
The effect of CSE and MEL on induced intracellular ROS of IEC-6 cells is shown in Figure 3c. Oxidation was induced by tBOOH 1 mM, which significantly increased the production of intracellular ROS ($p < 0.05$). As expected, vitamin C significantly reduced the formation of induced ROS ($p < 0.05$). When intestinal cells were pre-treated with different concentrations of CSE (0.004, 0.04 and 0.4 mg/ml), a significant reduction of induced ROS was observed ($p < 0.05$). The reduction of ROS levels by CSE has also been studied in other cell lines, such as liver and skin cells (Iriondo-DeHond *et al.*, 2019, Iriondo-DeHond *et al.*, 2016). Our results agreed with those described for these other cell lines. In addition, induced levels of ROS were also decreased when cells were pre-treated with MEL at 0.4 mg/ml. Results seem to indicate that low molecular weight compounds present in CSE were removed during the purification process, such as CGA, that might have contributed to the antioxidant effect observed on intestinal cells. To date, no other study has been found in the literature that has looked into the effect of MEL from coffee silverskin on induced intracellular ROS. Thus, the MEL fraction obtained from coffee silverskin in this study shows *in vitro* antioxidant capacity and is effective against induced ROS.


% Viability
140
120
100
80
60
40
20
0
c
a
d
c
c
a
de
cdef
cef
b
Control
DMSO
0.004
0.04
0.4
4
0.004
0.04
0.4
4
Control
CSE (mg/ml)
MEL (mg/ml)

(a)


% ROS
200
160
120
80
40
0
c
d
a
b
a
a
b
b
bc
Control
tBOOH
Vit C
0.004
0.04
0.4
0.004
0.04
0.4
Control
CSE (mg/ml)
MEL (mg/ml)

(b)



(c)

Figure 3. Effect of CSE and MEL on IEC-6 (a) cell viability, (b) physiological ROS and (c) induced intracellular ROS. Cells were cultured with different concentrations of CSE or MEL for 24 h. Then, the MTT assay was performed to determine cell viability and ROS were measured using the DCFH-DA probe. Control - untreated cells; death control - DMSO (50 %); oxidation control - tBOOH (1 mM); antioxidant control - vitamin C (10 µg/ml). Data are shown as the mean ± SEM of three independent experiments. Different letters above columns indicate significant differences among treatments (Tukey test, $p \leq 0.05$).

3.2.2 Toxicity and fiber effect

In this study, MEL was administered in the drinking water in a non-invasive manner as previously described. Previous studies have also administered samples in this way, for instance, freeze-dried instant coffee was administered to animals in a solution at 1.2 g/kg of body weight (Choi *et al*., 2011).

The mean weight of the animals at the beginning of the study was 186 ± 6.27 g (control group) and 174 ± 5.99 g (MEL group). Throughout four weeks of the *in vivo* study, there was a gradual increase in weight in both groups of animals, without showing statistically significant differences in this parameter due to the exposure of the animals to MEL in the drinking water (Table S1 shows final weight: control: 321.8 ± 8.5 g; MEL: 318.6 ± 16.4 g; $p > 0.05$). With regards to food intake, no statistically significant differences were observed either ($p > 0.05$). Food intake of the control group was 24.24 ± 0.95 g/day/rat and that of the group with MEL was 22.04 ± 0.96 g/day/rat. The group treated

with the MEL beverage presented an intake of 37.06 ± 2.33 ml/day/rat and the control group of 34.03 ± 1.14 ml/day/rat. A tendency of a higher liquid consumption in the group treated with the MEL beverage, compared to the control group was observed, but the difference was not statistically significant ($p > 0.05$). To date, there are no *in vivo* studies with coffee silverskin melanoidins. However, the effect of coffee beverage consumption *in vivo* has been addressed. Animals treated with the coffee beverage for 4 weeks showed lower body weight and food intake when compared to the control animals. This might be the result of decreased food intake, due to a loss of appetite in the animals with coffee beverage, since the consumption of caffeine, one of the major biologically active components of coffee, reduces energy intake by 22 % (Choi *et al.*, 2011). According to the literature on studies carried out with fructans with an inulin content of ≥ 90 %, rats treated with high fiber diet, three times per week for eight weeks, showed significantly lower consumption of food compared to the control group (Márquez-Aguirre *et al*., 2016; Rendón-Huerta *et al*., 2012). Castillo Andrade *et al*. (2018) described that this effect may be due to the Glucagon-like peptide type 1 (GLP-1) production that increases satiety and reduces energy intake. Authors also observed that the treated group showed higher water intake, which may be associated with high fiber consumption (Castillo Andrade *et al.*, 2018). Our results seem to indicate that the slight decrease in food consumption may be due to the presence of MEL, and its high fiber composition may explain the slight increase in drinking.

Throughout the study, no sign of clinical dehydration was found in any of the rats and the appearance of the perianal area as well as that of the fecal pellets were normal (Fukudome *et al.*, 2014). Table S1 shows the weights of the different organs normalized to the weight of the corresponding rat at sacrifice. Compared to control animals, in those treated with MEL there was a significant decrease in the weight of the small intestine, probably due to a concomitant decrease in its content (milking), which could be associated with an increased intestinal transit (see below). Even though most other organs showed no macroscopic modifications, the liver and kidneys presented slightly lower weight in MEL (although the difference was not statistically significant for the left kidney). No other study has been found in the literature that has looked into coffee silverskin melanoidins; however, studies on CSE, have found that CSE at 2 g/kg did not produce visible signs of toxicity nor did they find differences between the weights of the different organs ($p > 0.05$) (Iriondo-DeHond *et al.*, 2019). While liver and kidney weights were not measured in studies carried out in rats exposed chronically to a high fiber diet (fructans with an inulin content of ≥ 90%), the ability of hepatocytes to biosynthesize plasma proteins or renal tissues to remove waste substances was not

affected (Castillo Andrade *et al*., 2018; Márquez-Aguirre *et al*., 2016). The lack of major differences in the weight and appearance of the different organs suggests that exposure to MEL beverage does not produce a meaningful toxicity.

The fiber effect of melanoidins was specifically studied using radiographic analyses and bead expulsion test after 4 weeks exposure to MEL. Results of the radiographic study are shown in Figures 4 and 5. Results of the semiquantitative analysis of the stomach, for the control group, showed a progressive emptying of barium throughout the 8 hours of the radiographic session. Compared to control animals, the group treated with the MEL beverage showed statistically significant differences for time points 1 and 2 ($p < 0.05$), although this difference was possibly negligible from a clinical point of view (Figure 4a). The motility curves for the small intestine showed a filling phase (0 - 1 h), a plateau (1 - 2 h) and a progressive emptying phase (2 - 8 h); these were practically overlapping for both groups throughout the radiographic session, without showing any statistically significant difference at any time-point ($p > 0.05$) (Figure 4b). Figure 4c shows the motility curve for the caecum. In the filling phase, significant differences were observed ($p < 0.01$), since it took four hours for the caecum of the control animals to be completely filled up, whereas that of the animals treated with MEL beverage, was practically filled up in only two hours. Thereafter, the caecum remained practically full until the end of the study in both groups, with no statistically significant differences between them. With regards to the colorectum, the control group began to form fecal pellets two hours after contrast and reached the maximum score at 8 hours of the radiographic session (Figure 4d). The curve for the colorectum of MEL group overlapped with that of control animals, without any statistically significant difference ($p > 0.05$).

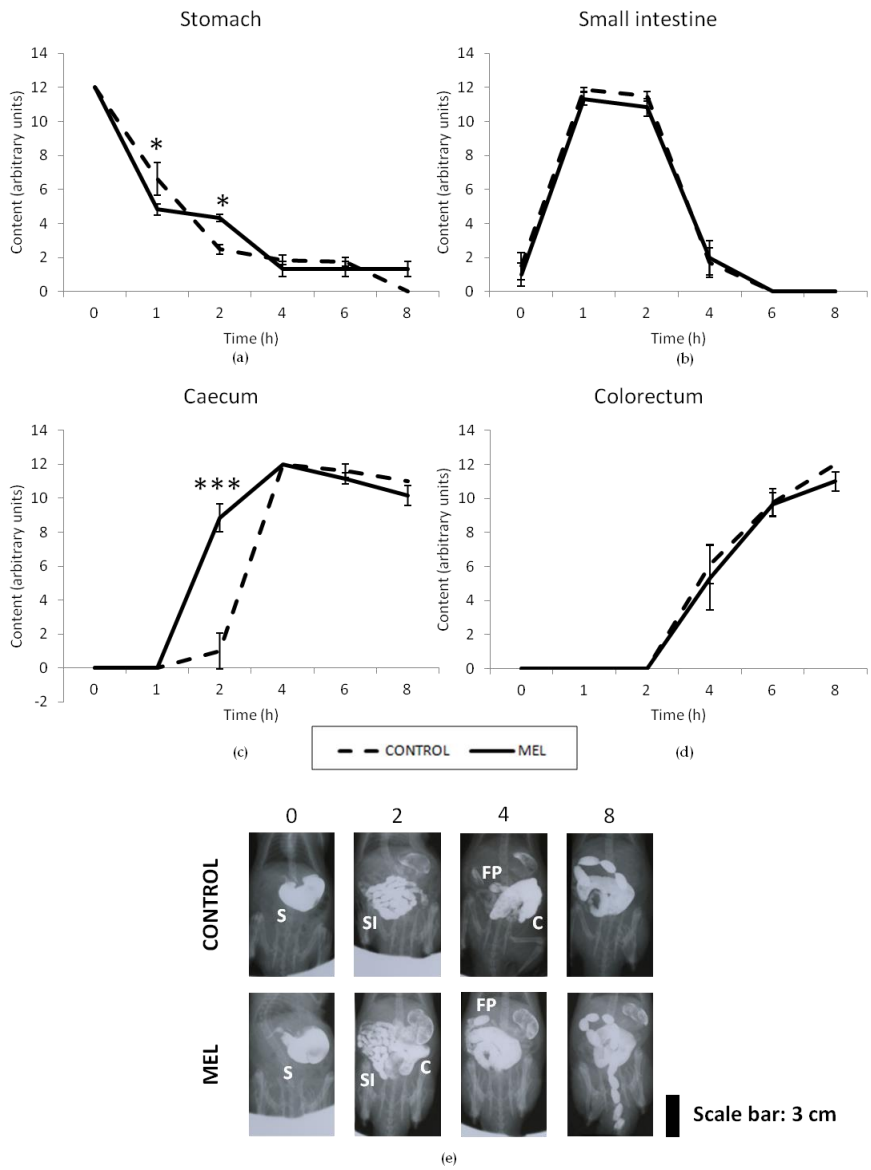


Figure 4. Radiological analysis of gastrointestinal motor function in rats - a semiquantitative analysis. A dose of barium sulfate (3 ml, 2 g/ml) was intragastrically administered at time 0, and X-rays were taken immediately and 1, 2, 4, 6 and 8 h after administration. Motility curves for the stomach (a), small intestine (b), caecum (c) and colorectum (d) were obtained from control rats (n = 8) and rats treated with MEL (4 g/L) (n = 6). Data represent the mean ± SEM. * $p < 0.05$, *** $p < 0.001$ (Two-way ANOVA followed by Bonferroni *post-hoc* test). Representative X-rays (e) obtained from a control and a MEL-treated rat at 0, 2, 4 and 8 h after administration of barium sulfate. S - stomach; SI - small intestine; C - caecum; FP - fecal pellets (within the colorectum).

The alterations in the size and density of stomach, caecum and fecal pellets are shown in Figure 5. No significant differences were found for morphometry and densitometry in the stomach between control and MEL groups ($p > 0.05$). Figures 5c and 5d show the curves for the caecum size and density, respectively. Significant differences were observed ($p < 0.01$) at 2 h. These results agreed with the semiquantitative results shown in the motility curves for the caecum (Figure 4c), as described above. The intense folding of the small intestine in the abdomen did not allow for differences to be seen in the semiquantitative study, but barium reached and filled the caecum much faster in the MEL group compared to the control group. This was not due to a faster gastric emptying (Figure 4a, 5a, 5b) or to a reduction in the length of the small intestine (Table S1).; most likely, the propulsive activity of small intestinal semisolid contents was increased by MEL. Additionally, the number of fecal pellets found within the colorectum was slightly bigger in the group treated with the MEL beverage than in the control group, at 4 - 8 h (at 4 h, the Student's t-test showed that this difference was actually significant) (Figure 5e). This suggests that pellet formation is accelerated by MEL (differences might be highlighted by including additional time points in the radiographic session, i.e., 3 h). Although no significant differences in the density of fecal pellets were found, they tended to be bigger in the MEL group than in the control group ($p = 0.1610$, Student's t-test; Figure 5e). These increased number and size of fecal pellets might be a result of the higher fiber intake in the MEL group, making them slightly more effective to mechanically stimulate the colon.

Previous studies have found that animals treated with fructans showed higher fecal production and a softer consistency compared to the control group (Castillo Andrade *et al.*, 2018). Increased fecal excretion has been related to fiber consumption and its stimulating effect on the absorption of water and electrolytes from the lumen of the colon, which leads to a greater fecal viscosity and a general decrease in intestinal transit time (Mudgil & Barak, 2013). In previous studies, the effect of coffee grounds of Robusta variety on gastrointestinal motor function was studied. It was administered by gavage at a dose of 1 g/kg once a day from Monday to Friday for four consecutive weeks. Compared to vehicle, coffee grounds increased transit after the first but not after the 14th and 28th administrations, suggesting the development of tolerance to the laxative effect (Martirosyan, 2011). In the present study, transit was increased even after 28 days of MEL exposure in drinking water. Further studies are warranted to determine the effects of MEL at different exposure times.

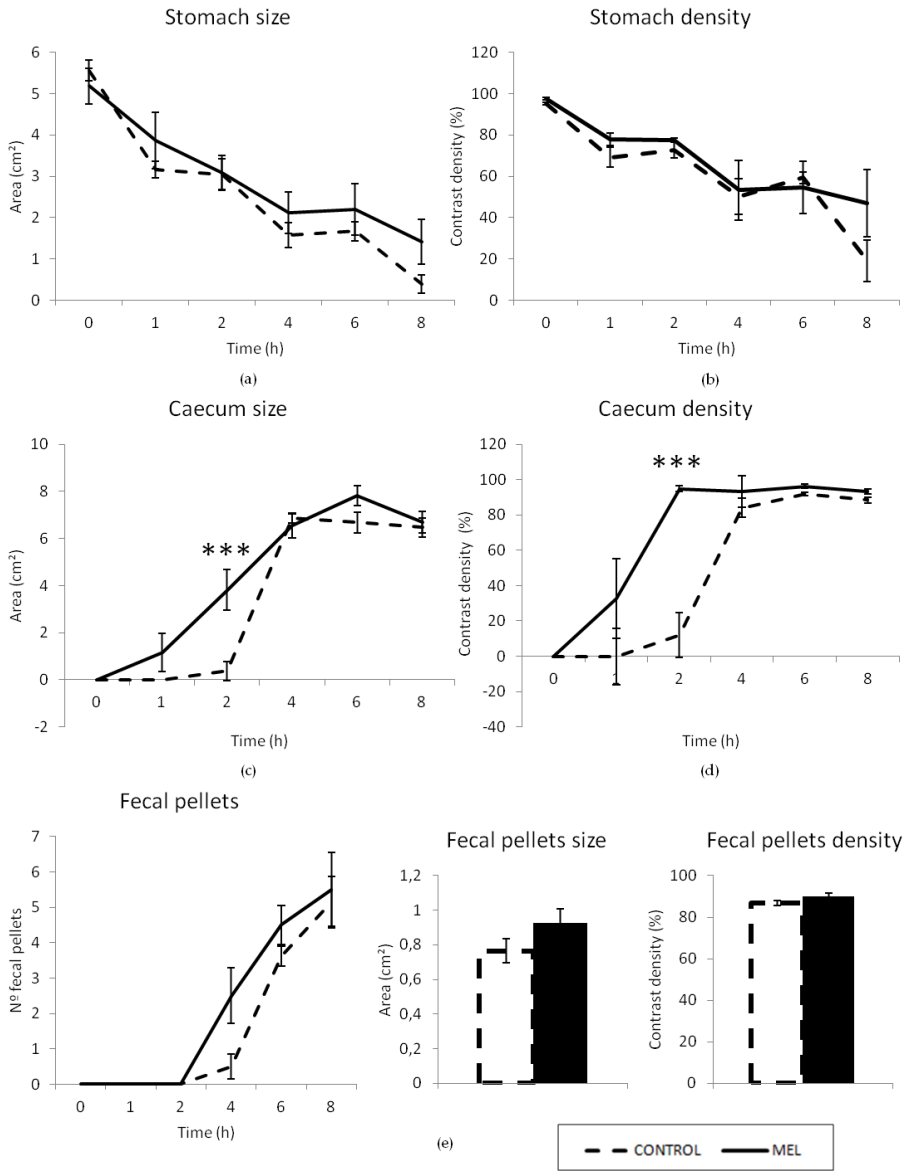


Figure 5. Radiological analysis of gastrointestinal motor function in rats - morphometric and densitometric study. A dose of barium sulfate (3 ml, 2 g/ml) was intragastrically administered at time 0 and X-rays were taken immediately and 1, 2, 4, 6 and 8 h after administration. Morphometry and densitometry analyses of the stomach (a, b), caecum (c, d) and fecal pellets (e) are shown for control rats (n = 8) and rats treated with MEL (4 g/L) (n = 6). Size and density were evaluated using Image J (see text for details). Data represent the mean ± SEM. *** $p < 0.001$. (Line graphs: Two-way ANOVA followed by Bonferroni post-hoc test. Bar graphs: Student's t-test).

There were no statistically significant ($p > 0.05$) differences in the lengths of the small intestine and colon between the control group and the MEL group (Table S1). Previous studies with fructans of *Agave salmiana* in Wistar rats for 35 days observed that the size and length of the caecum and colon were bigger with respect to the animals without treatment. This was considered a positive effect because it indicates a larger area available for the absorption of nutrients and the increase of the beneficial intestinal microbiota due to the consumption of fructan, and concluded that this fermentation began in the caecum (Castillo Andrade *et al*., 2018). More research is needed to determine if longer exposure to MEL might induce similar results.

Finally, the bead expulsion times were 470 ± 141 s for the control group and 502 ± 142 s for the MEL group, with no statistically significant differences between both groups ($p > 0.05$). In this test, the pellet was inserted only 3 cm into the colorectum from the anus, in a region where, under normal conditions, the fecal pellets are already formed and, possibly, the effect of the MEL beverage occurs in regions closer to the caecum, where the aqueous content of the feces can still be greatly altered. The fact that MEL beverage did not modify this parameter also indicated that the motor agents (intrinsic and extrinsic innervation, smooth muscle and interstitial cells of Cajal or pacemakers, (Furness, 2006)) involved in colonic propulsion, at this level are intact and respond similarly to the same mechanical stimulus (8 mm diameter-bead). This might not be the same for the small intestine, whose propulsive activity was increased as shown above, and more studies are needed to identify the specific mechanism involved. Whatever the case may be, no other study has been found on coffee, coffee silverskin or coffee silverskin melanoidins for this parameter.

4. Conclusions

This study provided novel information on the characterization of coffee silverskin melanoidins. Microbiological quality and health status of the treated animals support the food safety level of high molecular weight compounds, including melanoidins. The conditions for the isolation process were effective in obtaining a high molecular weight fraction (>10 kDa). The results of chemical, structural and functional characterization *in vitro*, showed the extraction of melanodins with antioxidant properties, which might be defined as the "Maillardized antioxidant dietary fiber". *In vivo* pilot study results of intestinal motility confirmed an acceleration of the intestinal transit in animals treated with MEL, even after 28 days of exposure. Coffee silverskin melanoidins have the potential to be used as a functional food ingredient.

Acknowledgments: The authors would like to thank SUPRACAFE for kindly providing the coffee silverskin. A. Iriondo-DeHond is a fellow of the FPI predoctoral program of MINECO (BES-2015-072191). Y. López-Tofiño thanks the Comunidad Autónoma de Madrid for her predoctoral contract (PEJD-2017-PRE/BMD-3924).

Funding: The SUSCOFFEE Project (AGL2014-57239-R) funded this research.

Author Contributions: M.D.C. and R.A. designed the study and S.T., A.I., T.H., M.P., F.V., M.D.C. and R.A. analyzed the data. S.T., A.I., T.H., M.D.C. and R.A. prepared the manuscript, whereas S.T and M.P. carried out separation of the macromolecular fraction by UF, S.T., A.I., T.H., F.V., C.G and Y.L., conducted the biological experiments. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

Abalo, R., Cabezos, P. A., López-Miranda, V., Vera, G., González, C., Castillo, M., … Martín, M. I. (2009). Selective lack of tolerance to delayed gastric emptying after daily administration of WIN 55,212-2 in the rat. *Neurogastroenterology and Motility*, *21*(9), 22–24. https://doi.org/10.1111/j.1365-2982.2009.01315.x.

Alves, R. C., Rodrigues, F., Nunes, M. A., Vinha, A. F., & Oliveira, M. B. P. P. (2017). State of the art in coffee processing by-products. In C. M. Galanakis (Ed.), *Handbook of Coffee Processing By-Products Sustainable Applications*. *Academic Press-Elsevier*. London, UK, 2017; pp. 1–26 ISBN 978-0-12-811290-8.

Bakondi, E., Gönczi, M., Szabó, É., Bai, P., Pacher, P., Gergely, P., … Virág, L. (2003). Role of intracellular calcium mobilization and cell-density-dependent signaling in oxidative-stress-induced cytotoxicity in HaCaT keratinocytes. *Journal of Investigative Dermatology*, *121*(1), 88–95. https://doi.org/10.1046/j.1523-1747.2003.12329.x.

Barbin, D. F., Felicio, A. L. de S. M., Sun, D. W., Nixdorf, S. L., & Hirooka, E. Y. (2014). Application of infrared spectral techniques on quality and compositional attributes of coffee: An overview. *Food Research International*, *61*, 23–32. https://doi.org/10.1016/j.foodres.2014.01.005.

Bekedam, E. K., Roos, E., Schols, H. A., Van Boekel, M. A. J. S., & Smit, G. (2008). Low molecular weight melanoidins in coffee brew. *Journal of Agricultural and Food Chemistry*, *56*(11), 4060–4067. https://doi.org/10.1021/jf8001894.

Borrelli, R. C., Esposito, F., Napolitano, A., Ritieni, A., & Fogliano, V. (2004). Characterization of a new potential functional ingredient: coffee silverskin. *Journal of Agricultural and Food Chemistry*, *52*(5), 1338–1343. https://doi.org/10.1021/jf034974x.

Broccardo, M., Guerrini, R., Petrella, C., & Improta, G. (2004). Gastrointestinal effects of intracerebroventricularly injected nociceptin / orphaninFQ in rats. *Peptides, 25,* 1013–1020. https://doi.org/10.1016/j.peptides.2004.03.015.

Cabezos, P. A., Vera, G., Castillo, M., Fernández-Pujol, R., Martín, M. I., & Abalo, R. (2008). Radiological study of gastrointestinal motor activity after acute cisplatin in the rat. Temporal relationship with pica. *Autonomic Neuroscience, 141*(1), 54–65. https://doi.org/10.1016/j.autneu.2008.05.004.

Castillo Andrade, A. I., Rivera Bautista, C., Godínez Hernández, C., Ruiz Cabrera, M. A., Fuentes Ahumada, C., García Chávez, E., & Grajales Lagunes, A. (2018). Physiometabolic effects of Agave salmiana fructans evaluated in Wistar rats. *International Journal of Biological Macromolecules, 108,* 1300–1309. https://doi.org/10.1016/j.ijbiomac.2017.11.043.

Charurin, P., Ames, J. M., & Del Castillo, M. D. (2002). Antioxidant activity of coffee model systems. *Journal of Agricultural and Food Chemistry, 50*(13), 3751–3756. https://doi.org/10.1021/jf011703i.

Choi, E. Y., Park, S. Y., & Cho, Y. O. (2011). Freeze-dried instant coffee can promote the activities of antioxidant enzymes and induce weight loss but also aggravate the plasma cholesterol profile in rats. *Nutrition, 27*(11–12), 1202–1205. https://doi.org/10.1016/j.nut.2011.02.003.

Dávalos, A., Bartolomé, B., & Gómez-Cordovés, C. (2005). Antioxidant properties of commercial grape juices and vinegars. *Food Chemistry, 93*(2), 325–330. https://doi.org/10.1016/j.foodchem.2004.09.030.

del Castillo, M. D., Ames, J. M., & Gordon, M. H. (2002). Effect of Roasting on the Antioxidant Activity of Coffee Brews. *Journal of Agricultural and Food Chemistry, 50*(13), 3698–3703. https://doi.org/10.1021/jf011702q.

del Castillo, M. D., Ibañez, M. E., Amigo, M., Herrero, M., Plaza del Moral, M., & Ullate, M. (2013). Application of products of coffee silverskin in anti-ageing cosmetics and functional food. Patent WO 2013/004873.

del Castillo, M. D., Martinez-Saez, N., Amigo-Benavent, M., & Silvan, J. M. (2013). Phytochemomics and other omics for permitting health claims made on foods. *Food Research International, 54*(1), 1237–1249. https://doi.org/10.1016/j.foodres.2013.05.014.

Echavarría, A. P., Pagán, J., & Ibarz, A. (2012). Melanoidins Formed by Maillard Reaction in Food and Their Biological Activity. *Food Engineering Reviews, 4*(4), 203–223. https://doi.org/10.1007/s12393-012-9057-9.

Esquivel, P., & Jiménez, V. M. (2012). Functional properties of coffee and coffee by-products. *Food Research International, 46*(2), 488–495. https://doi.org/10.1016/j.foodres.2011.05.028.

European Food Safety Authority (EFSA). (2015). Scientific Opinion on the safety of caffeine. *EFSA Journal, 13*(5), 1–21. https://doi.org/10.2903/j.efsa.2015.4102.

Fichna, J., Sałaga, M., Stuart, J., Saur, D., Sobczak, M., Zatorski, H., & Timmermans, J. (2014). Selective inhibition of FAAH produces antidiarrheal and antinociceptive effect mediated by endocannabinoids and cannabinoid-like fatty acid amides. *Neurogastroenterology & Motility, 26*, 470–481. https://doi.org/10.1111/nmo.12272.

Fichna, J., Schicho, R., Andrews, C. N., Bashashati, M., Klompus, M., Mckay, D. M., & Sharkey, K. A. (2009). Salvinorin A inhibits colonic transit and neurogenic ion transport in mice by activating j -opioid and cannabinoid receptors. *Neurogastroenterology & Motility, 21*, 1326-e128. https://doi.org/10.1111/j.1365-2982.2009.01369.x.

Fukudome, I., Kobayashi, M., Dabanaka, K., Maeda, H., Okamoto, K., Okabayashi, T., … Hanazaki, K. (2014). Diamine oxidase as a marker of intestinal mucosal injury and the effect of soluble dietary fiber on gastrointestinal tract toxicity after intravenous 5-fluorouracil treatment in rats. *Medical Molecular Morphology, 47*(2), 100–107. https://doi.org/10.1007/s00795-013-0055-7.

Furness, J. B. (2006). *The Enteric Nervous System*; Blackwell Publishing, Inc.: Malden, MA, USA, 2006; ISBN 978-1-4051-3376-0.

Garcia-Serna, E., Martinez-Saez, N., Mesias, M., Morales, F. J., & Castillo, M. D. Del. (2014). Use of Coffee Silverskin and Stevia to Improve the Formulation of Biscuits. *Polish Journal of Food and Nutrition Sciences, 64*(4), 243–251. https://doi.org/10.2478/pjfns-2013-0024.

Guglielmetti, A., Fernandez-Gomez, B., Zeppa, G., & del Castillo, M. D. (2019). Nutritional quality, potential health promoting properties and sensory perception of an improved gluten free bread formulation containing inulin, rice protein and bioactive compounds extracted from coffee byproducts. *Polish Journal of Food and Nutrition Sciences, 69*(2):157–166. https://doi.org/10.31883/pjfns-2019-0012.

Hashimoto, A., Sugimoto, Y., Suehara, K., & Kameoka, T. (2011). Influences of pH and temperature on infrared spectroscopic features of brewed coffee. *Procedia Food Science, 1*, 1132–1138. https://doi.org/10.1016/j.profoo.2011.09.169.

Hickman, D. L., & Swan, M. (2010). Use of a body condition score technique to assess health status in a rat model of polycystic kidney disease. *Journal of the American Association for Laboratory Animal Science : JAALAS, 49*(2), 155–159.

Hofmann, T., & Schieberle, P. (2002). Chemical interactions between odor-active thiols and melanoidins involved in the aroma staling of coffee beverages. *Journal of Agricultural and Food Chemistry*, *50*(2), 319–326. https://doi.org/10.1021/jf010823n.

Ii, F., Gussone, P., & Portici, E. (2004). Characterization of a New Potential Functional Ingredient : 1338–1343.

Iriondo-DeHond, A., Aparicio García, N., Velazquez Escobar, F., San Andres, M. I., Sanchez-Fortun, S., Blanch, G. P., … del Castillo, M. D. (2019). Validation of coffee by-products as novel food ingredients. *Innovative Food Science and Emerging Technologies*, *51*, 194–204. https://doi.org/10.1016/j.ifset.2018.06.010.

Iriondo-DeHond, A., Haza, A. I., Ávalos, A., del Castillo, M. D., & Morales, P. (2017). Validation of coffee silverskin extract as a food ingredient by the analysis of cytotoxicity and genotoxicity. *Food Research International*, *100*, 791–797. https://doi.org/10.1016/j.foodres.2017.08.012.

Iriondo-DeHond, A., Martorell, P., Genovés, S., Ramón, D., Stamatakis, K., Fresno, M., … Del Castillo, M. D. (2016). Coffee silverskin extract protects against accelerated aging caused by oxidative agents. *Molecules*, *21*(6), 1–14. https://doi.org/10.3390/molecules21060721.

Jiménez-Zamora, A., Pastoriza, S., & Rufián-Henares, J. a. (2015). Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties. *LWT - Food Science and Technology*, *61*(1), 12–18. https://doi.org/10.1016/j.lwt.2014.11.031.

López-Lorente, Á. I., & Mizaikoff, B. (2016). Recent advances on the characterization of nanoparticles using infrared spectroscopy. *TrAC - Trends in Analytical Chemistry*, *84*, 97–106. https://doi.org/10.1016/j.trac.2016.01.012.

Márquez-Aguirre, A. L., Camacho-Ruíz, R. M., Gutiérrez-Mercado, Y. K., Padilla-Camberos, E., González-Ávila, M., Gálvez-Gastélum, F. J., … Ortuño-Sahagún, D. (2016). Fructans from Agave tequilana with a Lower Degree of Polymerization Prevent Weight Gain, Hyperglycemia and Liver Steatosis in High-Fat Diet-Induced Obese Mice. *Plant Foods for Human Nutrition*, *71*(4), 416–421. https://doi.org/10.1007/s11130-016-0578-x.

Martirosyan, D.M. (2011) *Functional Foods and Chronic Diseases: Science and Practice*, Zhou, J. Ed.; *CreateSpace Independent Publishing Platform:* Scotts Valley, CA, USA, 2011; ISBN 9781976154966.

McRorie, J. W., & McKeown, N. M. (2017). Understanding the Physics of Functional Fibers in the Gastrointestinal Tract: An Evidence-Based Approach to Resolving Enduring Misconceptions about Insoluble and Soluble Fiber. *Journal of the Academy of Nutrition and Dietetics*, *117*(2), 251–264. https://doi.org/10.1016/j.jand.2016.09.021.

Mesías, M., Navarro, M., Martínez-Saez, N., Ullate, M., del Castillo, M. D., & Morales, F. J. (2014). Antiglycative and carbonyl trapping properties of the water soluble fraction of coffee silverskin. *Food Research International, 62*, 1120–1126. https://doi.org/10.1016/j.foodres.2014.05.058.

Mesías, M., & Delgado-Andrade, C. (2017). Melanoidins as a potential functional food ingredient. *Current Opinion in Food Science, 14*, 37–42. https://doi.org/10.1016/j.cofs.2017.01.007.

Moreira, A. S. P., Nunes, F. M., Simões, C., Maciel, E., Domingues, P., Domingues, M. R. M., & Coimbra, M. A. (2017). Transglycosylation reactions, a main mechanism of phenolics incorporation in coffee melanoidins: Inhibition by Maillard reaction. *Food Chemistry, 227*, 422–431. https://doi.org/10.1016/j.foodchem.2017.01.107.

Mudgil, D., & Barak, S. (2013). Composition, properties and health benefits of indigestible carbohydrate polymers as dietary fiber: A review. *International Journal of Biological Macromolecules, 61*, 1–6. https://doi.org/10.1016/j.ijbiomac.2013.06.044.

Murthy, P. S., & Naidu, M. M. (2012). Recovery of Phenolic Antioxidants and Functional Compounds from Coffee Industry By-Products. *Food and Bioprocess Technology, 5*(3), 897–903. https://doi.org/10.1007/s11947-010-0363-z.

Murthy, P. S., Naidu, M. M., & Srinivas, P. (2009). Production of α-amylase under solid-state fermentation utilizing coffee waste. *Journal of Chemical Technology and Biotechnology, 84*(8), 1246–1249. https://doi.org/10.1002/jctb.2142.

Napolitano, A., Fogliano, V., Tafuri, A., & Ritieni, A. (2007). Natural occurrence of ochratoxin A and antioxidant activities of green and roasted coffees and corresponding byproducts. *Journal of Agricultural and Food Chemistry, 55*(25), 10499–10504. https://doi.org/10.1021/jf071959+.

Nunes, F. M., & Coimbra, M. A. (2010). Role of hydroxycinnamates in coffee melanoidin formation. *Phytochemistry Reviews, 9*(1), 171–185. https://doi.org/10.1007/s11101-009-9151-7.

Oecd/Ocde; Ction, (1995). I. N. T. U. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Guidel. Test. Chem. 8, 1–8, doi:10.1787/9789264070684-en.

Oki, T., Nagai, S., Yoshinaga, M., Nishiba, Y., & Suda, I. (2006). Contribution of b-Carotene to Radical Scavenging Capacity Varies among Orange-fleshed Sweet Potato Cultivars. *Food Science and Technology Research, 12*, 156–160. https://doi.org/10.3136/fstr.12.156.

Ou, B., Hampsch-woodill, M., Prior, R. L., Laboratories, B., & Lane, T. (2001). Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using

Fluorescein as the Fluorescent Probe. *Journal of Agricultural and Food Chemistry*, *49*(10), 4619-4626. https://doi.org/10.1021/jf010586o.

Paiva, A., Ranocchia, K., Marques, M., da Silva, M. G., Alves, V., Coelhoso, I., & Simões, P. (2018). Evaluation of the quality of coffee extracts concentrated by osmotic evaporation. *Journal of Food Engineering*, *222*, 178–184. https://doi.org/10.1016/j.jfoodeng.2017.11.020.

Pérez-Hernández LM, Chávez-Quiroz K, Medina-Juárez LA, G. M. N. (2011). Compuestos fenólicos, melanoidinas y actividad antioxidante de cafe verde y procesado de las especies *Coffea arabica* y *Coffea canephora*. *Biotecnia*, *15*, 51–56. https://doi.org/10.18633/bt.v15i1.136.

Pourfarzad, A., Mahdavian-Mehr, H., & Sedaghat, N. (2013). Coffee silverskin as a source of dietary fiber in bread-making: Optimization of chemical treatment using response surface methodology. *LWT - Food Science and Technology*, *50*(2), 599–606. https://doi.org/10.1016/j.lwt.2012.08.001.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3.

Rendón-Huerta, J. A., Juárez-Flores, B., Pinos-Rodríguez, J. M., Aguirre-Rivera, J. R., & Delgado-Portales, R. E. (2012). Effects of Different Sources of Fructans on Body Weight, Blood Metabolites and Fecal Bacteria in Normal and Obese non-diabetic and Diabetic Rats. *Plant Foods for Human Nutrition*, *67*(1), 64–70. https://doi.org/10.1007/s11130-011-0266-9.

Ruíz, M. M., Nurgali, K., Uranga, J. A., Mosinska, P., & Fichna, J. (2018). Alterations of colonic sensitivity and gastric dysmotility after acute cisplatin and granisetron. *Neurogastroenterology & Motility*, *(August)*, 1–14. https://doi.org/10.1111/nmo.13499.

Sengar, G., & Sharma, H. K. (2014). Food caramels: a review. *Journal of Food Science and Technology*, *51*(9), 1686–1696. https://doi.org/10.1007/s13197-012-0633-z.

Silván, J. M., Morales, F. J., & Saura-Calixto, F. (2010). Conceptual study on maillardized dietary fiber in coffee. *Journal of Agricultural and Food Chemistry*, *58*(23), 12244–12249. https://doi.org/10.1021/jf102489u.

Wang, H. Y., Qian, H., & Yao, W. R. (2011). Melanoidins produced by the Maillard reaction: Structure and biological activity. *Food Chemistry*, *128*(3), 573–584. https://doi.org/10.1016/j.foodchem.2011.03.075.

Table S1. Effect melanoidin consumption on body weight, normalized rat organs weight (g/kg rat) and length of the small intestine and colorectum (cm) of control (n = 8) and treated rats (n = 6). Values represent the mean ± SEM. Asterisk denotes significant difference compared to control (T-student, * $p < 0.05$).

	Control	**MEL**
Body weight (g)	321.8 ± 8.5	318.6 ± 16.4
Stomach (g/kg)	12.1 ± 1.0	10.1 ± 1.0
Small intestine (g/kg)	33.2 ± 1.0	29.4 ± 1.0*
Milking[1] (g/kg)	8.4 ± 0.4	5.6 ± 0.6*
Caecum (g/kg)	17.6 ± 0.8	16.4 ± 1.9
Colon (g/kg)	15.0 ± 1.2	13.6 ± 3.3
Salivary glands (g/kg)	2.4 ± 0.1	2.2 ± 0.2
Left kidney (g/kg)	3.3 ± 0.08	3.1 ± 0.04
Right kidney (g/kg)	3.2 ± 0.03	3.05 ± 0.06*
Liver (g/kg)	38.8 ± 1.1	34.1 ± 1.0*
Epididymal fat (g/kg)	21.9 ± 2.3	20.2 ± 1.7
Pancreas (g/kg)	4.0 ± 0.3	4.3 ± 0.4
Small intestine (cm)	58.7 ± 1.7	58.3 ± 0.9
Colorectum (cm)	14.5 ± 0.6	14.8 ± 1.4

[1] Milking: content of the small intestine.

GENERAL DISCUSSION

1. Coffee cherry by-products generated in producing countries

1.1. Husk

The present PhD thesis provides novel data for supporting the valorization of coffee husk as a food ingredient for human consumption. Using a simple, low cost and environmentally friendly extraction process two different novel ingredients were generated (Figure 1): an enriched extract in bioactive compounds and a dietary fiber fraction (Chapter 1). But in order to use this coffee by-product for human consumption, special attention has to be taken regarding food safety. No pesticides were detected either in husk extract or in the insoluble fiber fraction. With regard to biological contaminants, aflatoxin B1 and enniantin B were not detected in any of the two novel ingredients. Low levels of ochratoxin A (OTA) were found in the insoluble fraction of husk (4.3 μg/kg), which were below the maximum levels of 5 μg/kg established by the European Commission (European Commission, 2005). However, OTA was not detected in husk extract. In addition, intake of an acute dose of 2 g/kg b.w. of coffee husk aqueous extract did not cause visible signs of toxicity, abnormal behavior or mortality. To the best of our knowledge, this is the first time data on contaminants and toxicity of husk have been published. Altogether, data ensure the food safety of novel ingredients derived from husk for human consumption.

The nutritional composition of the insoluble fraction of husk suggests its potential to be used as a food ingredient with the following nutrition claims: high in fiber and low in fat, given that this novel ingredient possesses more than 6 % of dietary fiber and less than 3 % fat (Chapter 1). Total dietary fiber (TDF) content reported in this fraction was 72 %, being 17 % of this fiber soluble. This novel ingredient also showed high antioxidant capacity *in vitro* (48.6 mg eq. CGA/g).

Husk aqueous extract contained 1.4 % of caffeine, 0.2 % of CGA and possessed antioxidant capacity *in vitro* (80.6 mg eq. CGA/g). Antioxidant properties of this extract at 1 mg/ml were also confirmed in human liver cells (HepG2). This is the first time that the antioxidant capacity of coffee husk extract is studied in human cellular models. This extract may be a potential agent for the prevention of cellular damage induced by oxidative stress.

Figure 1. Overview of coffee husk and parchment processing for their application as novel ingredients for human consumption or other applications of interest for the sustainability of the food industry.

Considering results obtained in this investigation and the inverted pyramid of food hierarchy proposed by the FAO (Introduction, page 32, Figure 1), the most preferred application for coffee husk is as two different food ingredients for human consumption. Other feasible applications of coffee husk are the production of biofuels, composts, animal feed and specific materials such as biosorbents, enzymes and chemicals (M. Dolores del Castillo et al., 2019). However, these applications do not make the most efficient use of this material and therefore, are less preferred for repurposing and recycling this coffee by-product.

Results derived from the present investigation suggest that husk generated in the wet processing obtained after the depulping step should be dried in the sun to reduce its moisture to 10 %, as it is already carried out by a Spanish coffee company (Supracafé S.A.). Different drying methods, thermal and non-thermal, should be evaluated in order to prevent the occurrence of the Maillard reaction during processing (Michalska, Honke, Łysiak, & Andlauer, 2016). Recent preliminary data carried out by our research group have shown the presence of Maillard reaction products, such as melanoidins, in coffee husk obtained by the wet processing and dried in the sun for 21 days. These high molecular compounds seem to be the main contributor to the color and antioxidant properties of this by-product (unpublished data). Traces of acrylamide (86 µg/kg), a food processing contaminant generated by the Maillard reaction, have been also found in husk; thus it is of great importance to search for alternative drying methods to mitigate the appearance of this undesirable compound and to ensure the food safety of the new materials.

After drying, the simple fractionation process carried out by aqueous extraction could be performed. The aqueous extract obtained from coffee husk enriched in phytochemicals might be used for techonological and health purposes. On the one hand, it could be used as a food preservative or as a food colorant. The natural food-colorant industry is growing 10 – 15 % annually, and awareness is increasing in developed countries about the harmful effects and consequences of using synthetic colorants. Coffee husk obtained from the wet coffee processing has been proposed as a potential source of anthocyanins for natural food colorants. In the wet process, coffee husk is removed before drying and its color is rapidly degraded by the action of enzymes or oxygen. As a result, large amounts of natural colorants are wasted (Hartati et al., 2012). Cyanidin 3-rutinoside was characterized as the most abundant anthocyanin, responsible for the red color observed in the outside of the fresh coffee berry (Murthy et al., 2012). Prata and Oliveira (2007) carried out the extraction of monomeric and polymeric anthocyanins in five varieties of coffee husk. There were no significant differences in

anthocyanin content among the studied varieties, and the average content of monomeric anthocyanins in husk was 19.2 mg of pigment per 100 g of fresh husk (Prata & Oliveira, 2007). An appropriate management of this by-product, as to the drying the process, is necessary to preserve these compounds. Coffee husk possesses great potential as an economical source of natural colorants.

On the other hand, the enriched extract obtained from coffee husk may be also used for healthy purposes. VDF FutureCeuticals Inc., a manufacturer of fruit, vegetable and grain ingredients, has been producing extracts from the coffee cherry for human health for over 10 years ("FUTURE CEUTICALS," 1999). These extracts are proposed to be used to improve human health, specifically brain health, as ready-to-drink beverages, supplements or functional snaks. NeuroFactor™ is a natural, patented extract of whole coffee fruit that contains a unique polyphenol profile that has been shown in a clinical study to stimulate the production of Brain-Derived Neurotrophic Factor (BDNF), a key neuroprotein involved in overall brain health. BDNF has been widely reported to play a critical role in neuronal development, maintenance, repair and protection against neuro-degeneration. Antioxidant properties of husk extract in human cellular models reported in the present investigation support the use of this extract in the prevention of cellular damage induced by oxidative stress. In this sense, coffee husk has been used to improve skin health, as a cosmetic ingredient, and it is said to be "skin-tightening, redness-soothing properties and with super-antioxidant powers". The application of coffee cherry extracts in skin-care products has been proposed by Miljkovic in 2011 in a US patent (Miljkovic, 2011). Nowadays, different skin-care products such as face creams, shampoos and body lotions containing extracts from coffee cherry are commercially available. Altogether, the aqueous extract obtained in this PhD thesis by a sustainable, low-cost and easy to scale-up process has great potential to be applied in food industry with both technological and health purposes.

The extraction waste, the insoluble fraction obtained after the aqueous extraction of coffee husk, can also be repurposed in the food industry sector (Figure 1). This insoluble fraction is also generated during the elaboration of the commercially available "cascara" beverages (Jelderks & Zimmermann, 2017). Recent studies carried out by our reseach group have used this insoluble fraction in gluten-free breads to improve their nutritional and sensory quality (Rios, 2018). The Coffee Cherry Co. has also developed flour from coffee husk and it proposes its application as a food ingredient in different food matrices such as bakery products, ice-cream and sauces ("The Coffee Cherry Co.," 2014). This company produces flour from the whole by-product, in contrast, we suggest to fraction

husk in two different ingredients to obtain more value and make a more efficient use of this coffee by-product.

1.2. Parchment

Aqueous extraction of parchment resulted in a very low extraction yield (2.3 %), therefore, it would be more appropriate to use it directly as one ingredient (Figure 1). Safety of this novel ingredient was confirmed in this investigation by the abscense of pesticides and mycotoxins (aflatoxin B1 and enniantin B). This by-product showed values of OTA (2.3 μg/kg) below the limit established by the European Commision (5 μg/kg) (European Commission, 2005). Microbiological risks should always be avoided, therefore, a stabilization is step fundamental to guarantee the microbiological and physicochemical stability of biomaterials (Torres-León et al., 2018). Safety evaluation was completed by an acute toxicity study, which showed no adeverse effects or mortality after the administration of 2 g/kg b.w. of parchment to the animals (Chapter 1). To the best of our knowledge, this is the first time safety of coffee parchment is evaluated by *in vivo* toxicity studies.

The insoluble fraction of prachment obtained after aqueous extraction presented high levels of insoluble dietary fiber (92 %). This fraction contained low levels of proteins (3 %) and fat (0.3 %). It possessed less antioxidant capacity than the other coffee by-products analyzed in this study, husk and silverskin.

High levels of caffeine (58 mg/g) and low values of CGA (6 mg/g) were obtained in the small amounts of aqueous parchment extract obtained. This extract presented high antioxidant capacity *in vitro* (202 mg eq. CGA/g) and also reduced intracellular oxidative stress in human liver cells. Aguilera *et al.* (2019) also carried out an aqueous heat assisted extraction to obtain an extract enriched in phenolic compounds from coffee parchment. Their results demonstrated the need of milling parchment to promote the extractability of phenolic compounds, such as CGA, vanillic acid, protocatechuic acid and *p*-coumaric acid (Aguilera, Rebollo-Hernanz, Cañas, Taladrid, & Martín-Cabrejas, 2019).

Altogether, results obtained from Study 1 (Chapter 1) suggest the use of parchment directly as a dietary fiber. A very recent study also proposes parchment as a promising low-calorie functional ingredient for dietary fiber enrichment in foods with hypoglycemic and hypolipidemic properties (Benitez et al., 2019). Results from this research showed that coffee parchment flakes possessed higher oil holding, water

holding, absorption and swelling capacities than parchment flour (Benitez et al., 2019). Previous preliminary studies carried out by our research group have evaluated the potential of coffee parchment fiber in gluten-free bread formulations. Enriched breads contained 6.25 % of parchment flour. The final gluten-free product had the nutrition claim "high in fiber" thanks to the supplementation with the coffee by-product flour and was accepted by consumers (Gonzalez, 2016). In addition, Cubero-Castillo *et al.* (2017) developed a cookie with 2 % of coffee parchment flour as a dietary fiber source and with antioxidant capacity. In this study, coffee parchment flour did not generate rejection on consumers which is a positive characteristic since dietary fiber from cereals has generally low acceptance (Cubero-Castillo, Bonilla-Leiva, & García-Velasques, 2017).

Considering the inverted pyramid of food lossess and waste repurposing (Introduction, page 32, Figure 1), parchment could be as an ingredient for human consumption. Thermal processing is the most extensively used method of flour preservation to destroy microorganisms and to extend its shelf-life. But thermal processing increases the digestibility of foods with loss of certain heat-labile nutrients thus lowering the nutritional value (Demir & Elgün, 2014). Therefore, other methods used for flour stabilization are ultraviolet-C or infrared stabilization and gamma radiation (Demir & Elgün, 2014; Deora, 2018). These non-thermal methods had a positive effect on the total phenolic content and antioxidant capacity of wheat flour (Demir & Elgün, 2014). For this reason, flour from parchment should be treated (thermally or non-thermally) to ensure its safety and extend its shelf-life, as it is a common practice for the preservation of other flours (Demir & Elgün, 2014) (Figure 1). According to the Spanish Association of Cereal Technicians, the aim of flour stabilization is to inactivate lipase for the elimination of bitter taste, increase shelf-life, reduce microbial load, eliminate anti-nutritional factors, improve sensory characteristics, increase fiber and control particle size (Fernandez-Vasallo, 2017).

Taking into account results derived from infrared spectroscopy studies that confirmed the presence of cellulose and lignin as the major components in parchment (Chapter 1), the best use of this by-product seems to be as a source of cellulose for other industrial uses (Figure 1). In accordance to that patented by Joseph Apuzzo, we propose the use parchment as a source of cellulose for the production of cellulose-based materials such as coffee filters or food packaging (Apuzzo, 2017). Sun-dried parchment obtained after the hulling step could be processed to be converted into sustainable materials. Cellulose film packaging material is being increasingly used personal care, food and beverage industries (Barrett, 2019). Lignocellulosic fibers and lignin are two of the most important natural bioresources in the world and show great potential to improve biodegradability

by replacing synthetic fibers in bioplastics. Lignin has showed the possibility to function as a plasticizer, stabilizer or bio-compatibilizer in bioplastics, making parchment a good candidate to be used in sustainable packaging (Yang, Ching, & Chuah, 2019).

2. Coffee beans processing by-products generated worldwide

2.1. Silverskin

Coffee silverskin (CS) is considered one of the main coffee industry residues produced in coffee consuming countries and supposes a pollution hazard when discharged in high amounts into the environment (Mussatto, Machado, et al., 2011). In the past years, our research group has focused its research in the valorization of this by-product and the present PhD thesis aims to obtain the scientific basis needed to apply for the authorization of CS as a novel food ingredient (Chapter 2). As for the by-products generated in coffee producing countries, we propose to carry out an aqueous extraction to CS generated during the roasting process of green coffee beans to generate two different food ingredients for human consumption: an aqueous extract enriched in nutrients and non-nutrient bioactive compounds (CSE) and an insoluble dietary fiber fraction.

To ensure food safety of ingredients obtained from CS for human consumption, the presence of chemical, biological and processing contaminants was studied (Chapter 1). CSE showed absence of pesticides and negligible levels of mycotoxins that were below the quatification limit (< 0.3 μg/kg). Pesticides, aflatoxin B1 and enniantin B were neither found in the insoluble fraction of CS, but OTA was detected in this fraction in lower levels than those allowed by the European Commission (2.9 μg/kg) (European Commission, 2005). With regard to processing contaminants, CSE presented 489 μg/kg of acrylamide which is formed during the roasting process. Since acrylamide is a highly soluble compound, it was found in CSE but not in its insoluble fraction. The value of acrylamide reported in CSE is near the amount established by the European Commission for roasted coffee (400 μg/kg) and below that established for instant coffee (850 μg/kg) (European Commission, 2017). Mitigation strategies recommended by the European Commission for coffee products should be carried out in order to reduce the content of this compound in CSE (European Commission, 2017). Controlling roasting conditions is one of the main mitigation strategies for reducing the content of acrylamide en coffee. Alternative roasting procedures, such as Instantaneous Controlled Pressure Drop (DIC)

process, should be considered for preventing the formation of this compound. This procedure consists in heating wet material in an autoclave for a short period of time with saturated steam or microwaves, followed by a rapid expansion to a final pressure lower than 10 kPa. DIC is proposed as an alternative procedure to obtain a better quality of roasted coffee (Kamal, Sobolik, Kristiawan, Mounir, & Allaf, 2008). Conditions set during this procedure might reduce the formation of acrylamide in CS during coffee and this novel ingredient would not contribute to the daily acrylamide intake. Further investigations are needed to confirm this hypothesis.

Further toxicological studies have been carried out for CSE during this PhD thesis. First, the absence of genotoxicity and cytotoxicity was confirmed when human liver cells (Hep G2) were treated with different concentrations of CSE (Chapter 2, Study 2). Our results also showed that CSE did not induce a significant increase of oxidation of purines and pyrimidines compared to the control. The study of the genotoxic potential is a basic component in the chemical risk assessment (Louis Bresson et al., 2016).

Regarding *in vivo* toxicity studies, single oral administration of CSE at 2 g/kg b.w. showed no signs of toxicity, abnormal behavior or mortality (Chapter 1, Study 1). In addition, repeated oral administration of CSE at a dose of 1 g/kg for a period of 28 days was also not toxic to the animals (Chapter 2, Study 3). None of the analyzed parameters indicated in the OECD Test Guidelines 407 for Repeated Dose 28-day Oral Toxicity Study in Rodents showed significant differences between treated and control rats. To the best of our knowledge, this is the first time the toxicity of CSE has been studied at three different levels, genomic, cellular and *in vivo*.

Considering the data obtained from the nutritional characterization of CSE (Chapter 2, Study 3), this extract has great potential for its use as a novel food ingredient. It can be considered a 'source of proteins', 'low in fat' and 'high in fiber' since it contains over 12 g/100 g of proteins, less than 3 g/100 g of fat and more than 6 g/100 g of dietary fiber, respectively (The European Parliament and The Council of the European Union, 2006). To the best of our knowledge, this is the first time that the amino acid profile of CSE has been reported and the amount of essential amino acids (34 – 37 %) makes it a good source of indispensable amino acids. The fatty acid profile and amount of soluble simple carbohydrates composing CSE was in accordance to that previously reported by other authors for CS (Bessada, Alves, Costa, Nunes, & Oliveira, 2018; A. S. G. Costa et al., 2018; Toschi et al., 2014). Considering the insoluble fraction of CS, it contained the remaining 70 % of dietary fiber, being most of it insoluble. This fraction also presented

10 – 20 % of proteins and around 3 % of fat, depending on the species. *In vitro* antioxidant properties were also reported in this fraction (Chapter 1).

With regard to the micronutrients composing CSE, this extract can also be considered a source of potassium, magnesium, calcium and vitamin C, since values of these micronutrients present in CSE represent a 15 % of the recommended allowance per 100 g of product (European Council, 1990). CSE is also a sustainable source of non-nutrient bioactive compounds such as CGA (9.4 mg/g) and caffeine (24 mg/g) (Chapter 1, Study 1).

The potential health promoting properties of CSE previously reported by our research group are reviewed in Annex 1. The present investigation has studied further health promoting properties. The antioxidant capacity of CSE was once again proved *in vitro* (Chapter 1) and this extract also showed antioxidant capacity at a genomic level (Chapter 2, Study 2). CSE protected against benzo(a)pyrene (B(a)P) induced DNA damage, strand breaks and oxidized purines and pyrimidines, in HepG2 cells. CSE showed higher protective effect against B(a)P-induced oxidative DNA damage compared to that observed for the strand breaks, possibly due to its well-known antioxidant capacity. Free CGA of linked to other chemical structures seemed to be a contributor of the observed chemoprotective effect of CSE. Antioxidant properties of CSE were also reported at a cellular level, physiological ROS levels were significantly reduced ($p < 0.05$) when human liver cells were treated with CSE at 1 mg/ml (Chapter 1, Study 1).

In healthy Wistar rats treated with CSE 1 g/kg b.w. for 28 days antioxidant biomarkers did not show differences compared to non-treated animals (Chapter 2, Study 3). Further antioxidant capacity analysis in different organs or other studies evaluating higher doses of CSE should be conducted. A previous *in vivo* study carried out by our research group demonstrated a specific chemo-protective effect of CSE in pancreatic tissue, probably associated with its antioxidant capacity (Fernandez-Gomez, Lezama, et al., 2016). Daily administration of CSE at 50 mg/kg b.w., for 35 days previous to the induction of diabetes with the diabetogenic agent streptozotocin reduced pancreatic oxidative stress and protein damage. Results derived from this study suggest that caffeine in CSE was metabolized and the metabolites protected the pancreas against oxidative stress (Fernandez-Gomez, Lezama, et al., 2016). Therefore, the use of CSE for the prevention and treatment of metabolic syndrome was patented by our research group in 2016 (WO/2016/097450, 2016).

The major component in CSE is dietary fiber (22 %). Fermentation of dietary fiber releases short chain fatty acids (SCFAs), which modulate genes and proteins involved

in anti-inflammatory processes (Meijer, De Vos, & Priebe, 2010). Fiber fermentability of CSE was demonstrated when total SCFAs significantly increased ($p < 0.05$) in feces of male rats treated with CSE (1 g/kg b.w.) for 28 days compared to male control animals (Chapter 2, Study 3). The prebiotic properties of CS have been previosuly described and have demonstrated that CS preferentially supports the bifidobacteria growth *in vitro*, suggesting that its consumption may have some prebiotic effects (Borrelli et al., 2004).

Altogether, we propose to apply the biorefinery approach, "a sustainable processing of biomass into a spectrum of marketable products and energy", to the CS generated during the roasting process (Imbert, 2017). An easy, sustainable and low-cost water extraction is suggested for the obtainment of two novel ingredients for human consumption: an enriched extract with potential health promoting properties and an insoluble fraction with antioxidant dietary fiber (Figure 2).

Based on its nutritional and chemical composition, the enriched aqueous extract could be used as a food ingredient for human consumption within the concept of a healthy diet to maintain health and well-being (World Health Organization (WHO), 2015). This would be a more efficient use and would add value to this by-product, which is in line with that described for food losses and waste recycling by the FAO (Introduction, Figure 1). CSE could be consumed as a food supplement or it could be added to the most suitable food matrix considering sensory acceptance of consumers. Our research group patented (Patent No. WO 2013/004873) in 2013 its use in food and cosmetic applications (María Dolores del Castillo et al., 2013). Studies regarding food applications in beverages (Martinez-Saez et al., 2014) or biscuits (Garcia-Serna et al., 2014), and the bioactivity of this extract as an anti-diabetic agent (Fernández-Gómez, 2016) have been previously published. This thesis provides novel information regarding the nutritional composition and safety aspects of CSE at different levels in order to be used as a food ingredient. This information is of great relevance to apply for the Novel Food Authorization following the Regulation (EU) 2015/2283 (Annex 2).

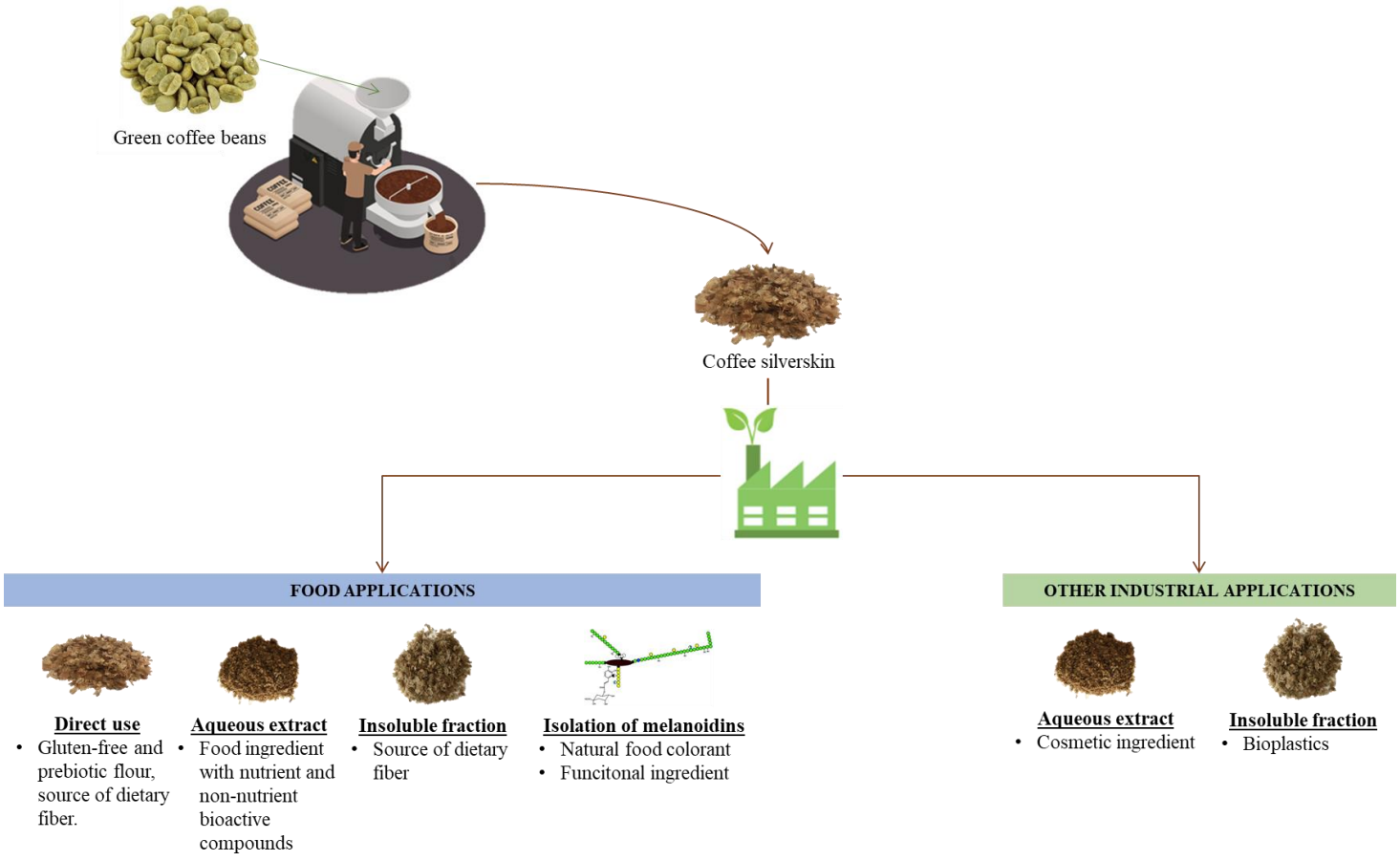


Figure 2. Sustainable strategy for the full recovery of coffee silverskin generated worldwide during the roasting process for human consumption and other industrial uses.

The potential of CSE as a cosmetic ingredient has also been studied in human skin cell lines and in *Caenorhabditis elegans*. CSE (1 mg/ml) significantly increased longevity in nematodes exposed to UVC compared to those cultured on a standard diet. In addition, CSE at 1 mg/ml increased resistance to skin cells exposed to tert-butyl hydroperoxide (t-BOOH) induced oxidative stress. The antiaging properties of CSE observed in this study may be due to its antioxidant character derived from phenolics (presumably caffeic acid) among other bioactive compounds present in the botanical material (Annex 3). Therefore, CSE extracts have the potential to be used as an antiaging ingredient in skin cosmetic products to reduce the oxidative stress and improve skin health. Taking into account the promising antioxidant and antiaging properties observed in this investigation, in 2017 CSE has been recognized as a cosmetic ingredient as "Water (and) Coffea Arabica/Robusta Chaff Extract" by the International Cosmetic Ingredient Nomenclature Committee (Annex 4).

The insoluble fraction remaining from the aqueous extraction of CS can also be used as a different food ingredient rich in dietary fiber with the final aim of recovering the whole by-product avoiding the generation of new wastes (Chapter 1). In previous studies, CS dietary fiber has been added to biscuits to improve their nutritional quality (Garcia-Serna et al., 2014). Another alternative for the use of CS insoluble fraction is for producing polylactic acid (PLA) as a replacement for synthetic polymers (Fahim, Chbib, & Mahmoud, 2019). The isolation and purification of cellulose from CS has been carried out and results showed the great potential of CS as a new source of cellulose for sustainable packaging (Alghooneh, Mohammad Amini, Behrouzian, & Razavi, 2017).

2.2. Spent coffee grounds

Spent coffee grounds (SCGs) are the other by-product distributed worldwide generated after the brewing process. Previous research has studied the potential of this by-product as a food ingredient (Rocio Campos-Vega et al., 2015; PCT/ES2014/070062, 2014; Martinez-Saez, Tamargo García, et al., 2017). New information on safety, nutritional value and bioactivity of SCGs is reported in the present PhD thesis.

Food safety of SCGs was evaluated by the determination of mycotoxins (Chapter 3). Aflatoxin B1 and enniantin B were not found and OTA levels (2.3 μg/kg) were under that established by the European Commission (European Commission, 2005). In addition, a single oral administration of SCGs at 2 g/kg b.w. resulted in no visible signs of toxicity, abnormal behavior or mortality. Neither the repeated intake of SCGs at a

dose of 1 g/kg b.w. for 28 days was noxious to the animals. To date, no other studies have analyzed mycotoxins or the *in vivo* toxicity of SCGs.

This coffee by-product is considered a source of dietary fiber (PCT/ES2014/070062, 2014). The second main component present in SCGs is fat. Lipid content of SCGs was 22 %, being palmitic (62.58 mg/g), stearic (14.76 mg/g), oleic (20.38 mg/g), linoleic (87.36 mg/g), and arachidic (6.68 mg/g) acids the main fatty acids found in this by-product. A total of 56 % of the total fatty acids were unsaturated. Higher amounts of polyunsaturated fatty acids (PUFAs) than saturated fatty acids (SFAs) in oil is recognized as being more positive for human health (Acevedo et al., 2013). In addition, the PUFA/SFA ratio >1 is positive for the reduction of serum cholesterol and atherosclerosis and prevention of heart diseases (Rocio Campos-Vega et al., 2015). In the present PhD thesis the bioaccessibility, metabolism and physiological effects related to this lipid fraction were also evaluated.

A total of 77 % of unsaturated fatty acids and low amounts of kahweol (7.09 µg/g) and cafestol (414.39 µg/g) were bioaccessible after *in vitro* digestion of SCGs. A significantly lower ($p < 0.1$) accumulation of lipids in the liver and a higher excretion of them in feces was found in rats treated with SCGs for 28 days. In addition, SCGs acutely accelerated intestinal motility in rats. Therefore, SCGs might be considered a sustainable, safe and healthy food ingredient with potential for preventing hepatic steatosis due to their effect as dietary fiber with a high fat-holding capacity. Studies suggest that insoluble fiber may play an important role for weight loss during consumption of a high-fat diet by adhering fat to its complex structure, and it has been inversely associated with the risk of type 2 diabetes (Du et al., 2010; Meyer et al., 2000).

In Europe, most of the SCGs are currently being incinerated or disposed of in landfills (Mata, Martins, & Caetano, 2018). The economic and environmental costs of disposing of SCGs are undesirable, for this reason alternative applications for repurposing SCGs are needed (McNutt & He, 2019). The potential applications for SCGs, from the most to the least preferred option considering the food waste hierarchy proposed by the FAO (Introduction, Figure 1), that are currently being researched are as follows:

1. **Nutraceuticals**. SCGs have been proposed as a protective agent against the onset and of chronic inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis. This protective effect is associated to metabolites produced by colonic fermentation of SCGs (SCFAs), which exhibited strong antiinflammatory potential by suppressing nitric oxide production and inhibiting

inflammatory mediators such as IL-10, CCL-17, CXCL9, IL-1β, and IL-5 cytokines (López-Barrera et al., 2016).

2. **Food ingredient**. Our research group has patented the use of SCGs as a food ingredient rich in antioxidant diertary fiber for bakery products. This ingredient could be directly applied in the manufacture of pastry and confectionery foods such as bread, cookies, and breakfast cereals, among others, making it a simple, low-cost alternative (PCT/ES2014/070062, 2014; Martinez-Saez, Tamargo García, et al., 2017).
3. **Novel beverages**. A distilled beverage with a coffee aroma has been developed by aqueous extraction of aromatic compounds from SCGs, supplemented with sugar and the production of ethanol (Sampaio et al., 2013).
4. **Food preservative**. The addition of SCGs to meat and other foods has been shown to provide antioxidant properties inhibiting lipid oxidation, and also antimicrobial properties reducing pathogenic bacterial growth and, therefore, spoiling of food (Jiménez-Zamora et al., 2015).
5. **Skincare products**. An emulsion containing 35 % of oil extracted from SCGs presented promising characteristics as a sunscreen. This formulation is industrial-scalable and suitable for topical use according to the rheological, mechanical and safety assessment (Marto et al., 2016).
6. **Animal feed**. SCGs may be used as an alternative to conventional feed ingredients because of their nutrient composition and relatively low cost (Seo, Jung, & Seo, 2015).
7. **Biodiesel.** It is one of the most popular research topics surrounding SCGs for energy use. It consists of first extracting the oils present in SCGs, and transesterifying them into Fatty Acid Methyl Esters (FAME), commonly referred to as biodiesel (Rocha et al., 2014).
8. **Bioethanol**. The oil-free SCGs from biodiesel production can be reused as a source of carbohydrates for ethanol production by fermentation (Rocha et al., 2014).
9. **Solid biofuel**. SCGs can be used alone or mixed with other biomasses such as pine sawdust and then this mixture can be submitted to pelletization. Pellets produced from SCGs were comparable to other biomass materials, but still had higher particle emissions than alternatives such as pure sawdust (Limousy, Jeguirim, Labbe, Balay, & Fossard, 2015).
10. **Composting and fertilizer**. Direct application of SCGs to soils has been found to be damaging due to its high C/N ratio, phenol content and acidity. Positive

results have been obtained from studies on the effect of mixing SCGs with other organic wastes in different ratios (Ronga, Pane, Zaccardelli, & Pecchioni, 2016).

11. **Materials for construction industry**. SCGs have been mixed with other waste materials such as recycled glass, bagasse ash or fly ash in order to produce materials with high compressive strengths, suitable for use as a subgrade material (Arulrajah, Kua, Horpibulsuk, Mirzababaei, & Chinkulkijniwat, 2017).
12. **Bioplastics**. Triglycerides extracted from SCGs using a green chemistry approach, based on supercritical CO_2 extraction, seem to be promising candidates for the production of bioplastics (Williamson & Hatzakis, 2019).
13. **Adsorbent of contaminants**. SCGs have been proven to be an effective adsorbent for a wide range of contaminants such as metal ions, dyes and bioactive compounds present in water (McNutt & He, 2019).

SCGs are currently being underutilized but considerable amount of research is being conducted in this field. Some of these applications have led to the commercialization of different products. For instance, the German company Kaffeeform reuses SCGs from six servings of coffee to make a coffee cup and saucer ("KAFFEE FORM," 2015). SCGs are also being commercially used in the production of sustainable yarn for clothing and for the elaboration of coffee-infused recycled polyester socks for odor control (Chen, 2009; Massachusetts Institute of Technology, 2010). A UK-based green energy firm, Bio-bean, recycles SCGs and turns them into "Coffee Logs" as a solid biofuel for multi-fuel stoves or to be used in barbecues (Bio-bean, 2013). Another English company, Greencup, transforms 200 - 300 tons of SCGs each year into 100% organic fertilizer (Fairweather, n.d.). Interestingly, in UK, Jeweller Rosalie McMillan creates pieces containing 70 % recycled coffee grounds, collected mostly from offices in London (McMillan, 2014). The most predominant commercially available SCGs based products are materials used for different applications. The present PhD thesis supports the use of SCGs as a food ingredient to make a more efficient use of this by-product and add more value to it.

Whatever the application, to be useful, the product has to loose great part of its moisture content. In the soluble coffee industry, for each ton of final product, 0.91 ton of SCGs are produced with a moisture level of 55 - 80 % (Gómez-De La Cruz, Cruz-Peragón, Casanova-Peláez, & Palomar-Carnicero, 2015). For higher burning efficiency of biomass or to ensure food safety, it is important to have significantly reduced the moisture level of SCGs (Corrêa, Santos, Fonseca, & Carvalho, 2014). Different drying procedures have been proposed to reduce moisture in SCGs, such as rotary drum dryer or cyclonic dryer (Cherry, 1974; Corrêa et al., 2014).

Altogether, we suggest to give different applications to SCGs depending on their origin (Figure 3). SCGs from the instant coffee industry, which has a controlled and standardized production process, may be directly used wet or dry as a food ingredient for human consumption. Special attention must be paid to the appearance of biological contaminants during the storage of SCGs, thus a drying step should be added in the repurposing process. SCGs generated in local restaurants or cafeterias (non-industrial) may be used for other purposes such as production of biofuels or biomaterials, also after a drying step. In this case, there would be no need to manage the by-product so carefully. The large amount of SCGs produced worldwide require several applications to diversify and contribute to the sustainability of the coffee industry. This investigation aimed to contribute to achieve this goal.

Figure 3. Five proposed applications for industrial and non-industrial spent coffee grounds (SCGs).

The consumption of new products within the European Union is a challenge in all countries, due to the need to ensure their safety. As the Regulation (EU) 2015/2283 states, "the free circulation of healthy and safe food products is an essential aspect of the domestic market as it greatly contributes to the health and well-being of the European citizens, as well as being in Europe's social and economic interest". This Regulation gives guidance to food businesses to easily bring new and innovative foods to the EU market, while maintaining a high level of food safety for European consumers (Diaz, 2019). This regulation should be followed in order to use coffee by-products as novel food ingredients.

The procedure of the Novel Food application starts whenever an applicant who is responsible for the introduction of a specific Novel Food sends an application to the European Commission (EC) (Figure 4). This will be included directly by the EC in the published list of applications and there is no intervention of any Member State during the application evaluation process. Then, the EFSA gives its opinion on the possible effects of the novel food on human health. The EFSA shall adopt its opinion within nine months from the date of receipt of a valid application. Within seven months from the date of publication of the EFSA's opinion, the Commission shall submit a draft authorising the placing on the market within the Union of a novel food and updating the Union list (The European Parliament and The Council of the European Union, 2015). The Union List of Authorized Novel Foods is a positive list containing all authorized novel foods added by means of Commission Implementing Regulations. Once a novel food is added to the Union list, it is automatically considered as being authorized and it can be placed in the European Union market.

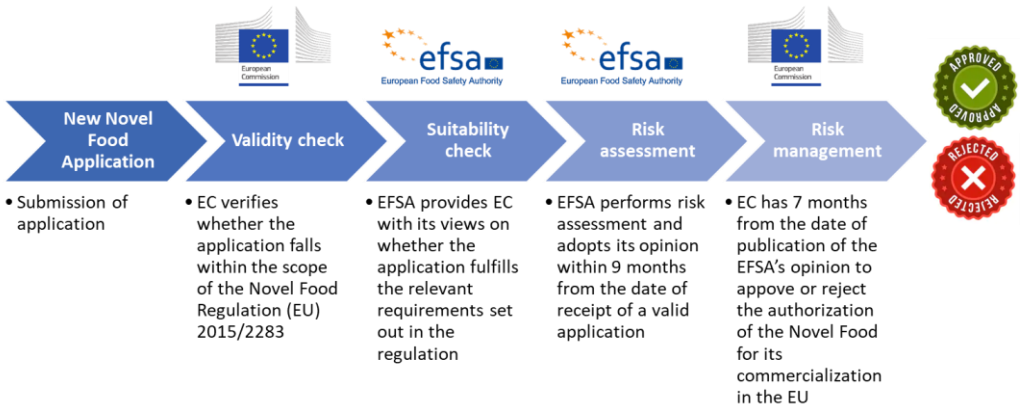


Figure 4. Novel Food Authorization workflow.

2.3. Isolated biomolecules from coffee by-products

Coffee melanoidins are being proposed as biomolecules possessing several health promoting properties (Mesías & Delgado-Andrade, 2017). Therefore, the high molecular weight fraction enriched in melanoidins was isolated from by-products generated after the roasting process, CS and SCGs. These by-products might be a sustainable alternative source of melanoidins (Mesías & Delgado-Andrade, 2017). Two extracts enriched in melanoidins and free of acrylamide were obtained from CS and SCGs by ultrafiltration (> 10 kDa) (Chapter 4, Study 5). Structural analyses identified melanoidins in the high molecular weight fraction of both by-products, and SCGs spectrum was also composed by a protein component. Both extracts showed antioxidant capacity *in vitro* and proteted human colon cells against induced oxidative stress.

To facilitate the structural and functional characterization of melanoidins from CS, we focused our attention on the optimization of the isolation process of melanoidins from this by-product (Chapter 4, Study 6). In this study, additional washing steps, diafiltration, were performed after ultrafiltration, low molecular weight compounds such as caffeine and chlorogenic acid could be eliminated. The isolated melanoidin fraction (MEL) was mainly composed by dietary fiber (75 %), melanoidins (15 %), proteins (2 %) and low levels of chlorogenic acids (0.2 %) and caffeine (0.1%). Such low caffeine content in MEL does not limit its use as a food ingredient in any population group (European Food Safety Authority (EFSA), 2015). Results suggest that MEL is mainly composed of polysaccharides, which is in accordance to that described for melanoidins of coffee beverages (Moreira et al., 2017).

With regard to the bioactivity of MEL obtained in this study, it showed antioxidant capacity *in vitro* and was effective against induced oxidative stress in rat intestinal cell lines. In addition, the fiber effect was confirmed *in vivo* when rats treated with MEL for 28 days showed an acceleration of the intestinal transit compared to control animals (Chapter 4, Study 6).

The potential role of dietary fiber in the prevention of diet-related chronic diseases is linked to the production of SCFAs from their fermentation by colonic bacteria, which can contribute to regulating the secretion of gastrointestinal hormones involved in satiety and appetite control, such as glucagon-like peptide-1 (GLP-1) and ghrelin (Barczynska et al., 2015; Martinez-Saez, Hochkogler, et al., 2017; Sleeth, Thompson, Ford, Zac-Varghese, & Frost, 2010; St-Pierre et al., 2009). These effects have been described in *in*

vivo studies in animal models (Delzenne, Cani, Daubioul, & Neyrinck, 2005) and human intervention trials (Tarini & Wolever, 2010).

Besides employing these biomolecules for nutritional and health (dietary fiber effect) purposes, melanoidins have also the potential to be used as a natural food colorant with antioxidant properties (Garcia-Serna et al., 2014; Guglielmetti, Fernandez-Gomez, Zeppa, & del Castillo, 2019). The use of caramel colorants accounts for more than 80 % by weight of colorants used in the food manufacturing industry (Zhang, Tao, Wang, Chen, & Wang, 2015). Melanoidins may be a sustainable alternative food colorant with health promoting properties.

In summary, data from this PhD thesis validate the use of the four coffee by-products in the food industry to contribute to a sustainable nutrition and health for the global population. The most preferred application of husk is for human consumption used directly or recovered as two ingredients: an aqueous extract enriched in bioactive compounds and a gluten-free fiber (Figure 5). However, parchment is mainly proposed as a material for sustainable and intelligent food packaging. The antioxidant insoluble fiber present in parchment is an excellent candidate to replace plastic and to preserve food during its storage. In order to avoid fires caused by the combustion of coffee silverskin in the roasters, this by-product can be removed, milled and used directly as a gluten free flour or as a material for plastic replacement. The limitation of using CS directly as a flour is the sensorial acceptance (smoked flavor) of the final product. Therefore, studies from this investigation propose the full recovery of CS in two novel sustainable and safe food ingredients obtained by aqueous extraction (bioactive extract and fiber fraction). This by-product is also a source of bioactive biomolecules, melanoidins, with potential to be used as a natural colorant or as an ingredient with health promoting properties, such as dietary fiber effect. Finally, industrial SCGs are also proposed to be used for human consumption while non-industrial SCGs may be repurposed in in other industrial uses.

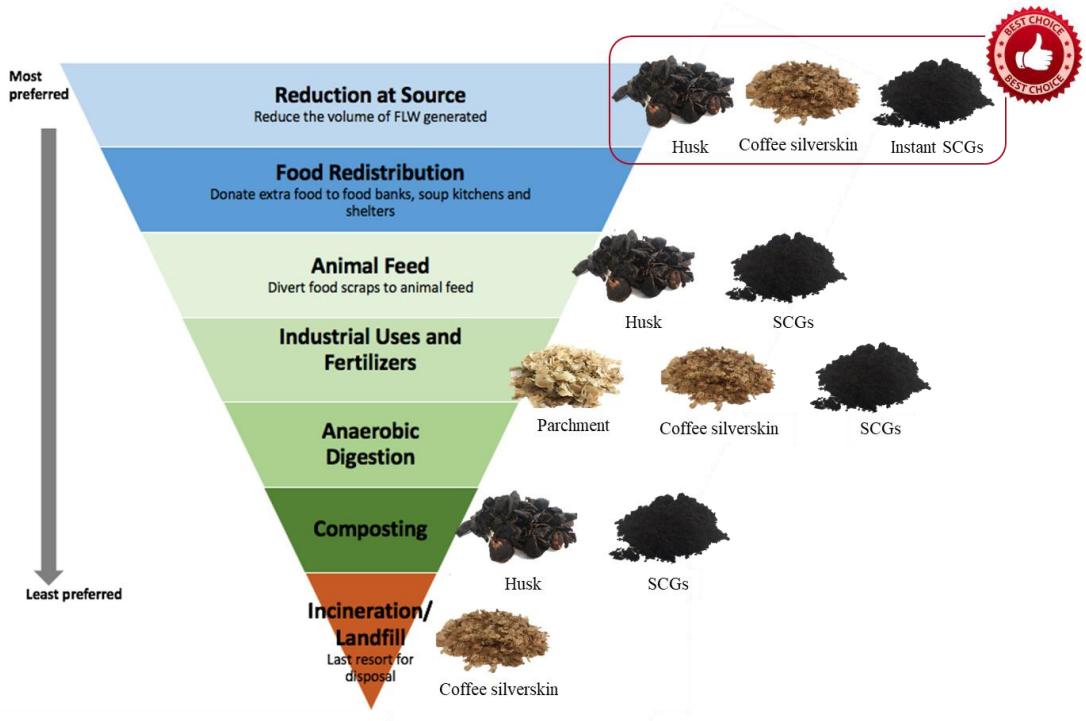


Figure 5. The most preferred applications for coffee by-products to contribute to the sustainability of the coffee industry, to reduce their environmental impact and to achieve a sustainable nutrition and health for the global population.

3. References

Acevedo, F., Rubilar, M., Scheuermann, E., Cancino, B., Uquiche, E., Garcés, M., … Shene, C. (2013). Spent coffee grounds as a renewable source of bioactive compounds. *Journal of Biobased Materials and Bioenergy*, *7*(3), 420–428. https://doi.org/10.1166/jbmb.2013.1369

Aguilera, Y., Rebollo-Hernanz, M., Cañas, S., Taladrid, D., & Martín-Cabrejas, M. A. (2019). Response surface methodology to optimise the heat-assisted aqueous extraction of phenolic compounds from coffee parchment and their comprehensive analysis. *Food & Function*, (C). https://doi.org/10.1039/c9fo00544g

Alghooneh, A., Mohammad Amini, A., Behrouzian, F., & Razavi, S. M. A. (2017). Characterisation of cellulose from coffee silverskin. *International Journal of Food Properties*, *20*(11), 2830–2843. https://doi.org/10.1080/10942912.2016.1253097

Alves, R. C., Rodrigues, F., Nunes, M. A. A., Vinha, A. F., & Oliveira, M. B. P. P. (2017). State of the art in coffee processing by-products. In C. Galanakis (Ed.), *Handbook of Coffee Processing By-Products: Sustainable Applications* (pp. 1–26). Academic Press-Elsevier.

Andrade, K. S., Gonçalvez, R. T., Maraschin, M., Ribeiro-do-Valle, R. M., Martínez, J., & Ferreira, S. R. S. (2012). Supercritical fluid extraction from spent coffee grounds and coffee husks: antioxidant activity and effect of operational variables on extract composition. *Talanta*, *88*, 544–552. https://doi.org/10.1016/j.talanta.2011.11.031

Apuzzo, J. (2017). Cellulosic composition containing coffee parchment cellulose and uses thereof.

Arulrajah, A., Kua, T.-A., Horpibulsuk, S., Mirzababaei, M., & Chinkulkijniwat, A. (2017). Recycled glass as a supplementary filler material in spent coffee grounds geopolymers. *Construction and Building Materials*, *151*, 18–27. https://doi.org/10.1016/J.CONBUILDMAT.2017.06.050

Ateş, G., & Elmacı, Y. (2018a). Coffee silverskin as fat replacer in cake formulations and its effect on physical, chemical and sensory attributes of cakes. *Lwt*, *90*, 519–525. https://doi.org/10.1016/j.lwt.2018.01.003

Ateş, G., & Elmacı, Y. (2018b). Physical, chemical and sensory characteristics of fiber-enriched cakes prepared with coffee silverskin as wheat flour substitution. *Journal of Food Measurement and Characterization*, *0*(0), 0. https://doi.org/doi.org/10.1007s11694-018-9988-9

Avallone, S., Guiraud, J.-P. J.-P., Guyot, B., Olguin, E., & Brillouet, J.-M. J.-M. (2000). Polysaccharide Constituents of Coffee-Bean Mucilage. *Journal of Food Science*, *65*(8), 1308–1311. https://doi.org/10.1111/j.1365-2621.2000.tb10602.x

Baiano, A. (2014). Recovery of biomolecules from food wastes - A review. *Molecules*, *19*(9), 14821–14842. https://doi.org/10.3390/molecules190914821

Ballesteros, L. F., Teixeira, J. a., & Mussatto, S. I. (2014). Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. *Food and Bioprocess Technology*, *7*(12), 3493–3503. https://doi.org/10.1007/s11947-014-1349-z

Ballesteros, L. F., Teixeira, J. A., & Mussatto, S. I. (2017). Extraction of polysaccharides by autohydrolysis of spent coffee grounds and evaluation of their antioxidant activity. *Carbohydrate Polymers*, *157*, 258–266. https://doi.org/10.1016/j.carbpol.2016.09.054

Barczynska, R., Bandurska, K., Slizewska, K., Litwin, M., Szalecki, M., Libudzisz, Z., & Kapusniak, J. (2015). Intestinal microbiota, obesity and prebiotics. *Polish Journal of Microbiology*, *64*(2), 93–100.

Barrett, A. (2019). Cellulose Film Packaging Gains Popularity with Plastic Reduction Initiatives. Retrieved from https://bioplasticsnews.com/2019/02/22/cellulose-film-packaging-gains-popularity-with-plastic-reduction-initiatives/

Bekalo, S. A., & Reinhardt, H.-W. W. (2010). Fibers of coffee husk and hulls for the production of particleboard. *Materials and Structures*, *43*(8), 1049–1060. https://doi.org/DOI 10.1617/s11527-009-9565-0

Belitz, H.-D., Grosch, W., & Schieberle, P. (2009). Coffee, tea, cocoa. In *In: Food Chemistry. Eds. Belitz, H.-D., Grosch, W. and Schieberle, P., 4th edition, Springer, Leipzig* (pp. 938–951).

Benitez, V., Rebollo-Hernanz, M., Hernanz, S., Chantres, S., Aguilera, Y., & Martin-Cabrejas, M. A. (2019). Coffee parchment as a new dietary fiber ingredient: Functional and physiological characterization. *Food Research International*, *122*(December 2018), 105–113. https://doi.org/10.1016/j.foodres.2019.04.002

Bessada, S. M. F., Alves, R. C., Costa, A. S. G., Nunes, M. A., & Oliveira, M. B. P. P. (2018). Coffea canephora silverskin from different geographical origins: A comparative study. *Science of the Total Environment*, *645*, 1021–1028. https://doi.org/10.1016/j.scitotenv.2018.07.201

Bessada, S. M. F., Alves, R. C., Oliveira, M. B. P. P., & Applications, C. (2018). Coffee Silverskin : A Review on Potential Cosmetic Applications. *Cosmetics*, *5*(1), 5. https://doi.org/10.3390/cosmetics5010005

Bio-bean. (2013). Coffee Logs. Retrieved from https://www.bio-bean.com/coffee-logs/#hero

Borrelli, R. C., Esposito, F., Napolitano, A., Ritieni, A., & Fogliano, V. (2004). Characterization of a new potential functional ingredient: coffee silverskin. *Journal of Agricultural and Food Chemistry*, *52*(5), 1338–1343. https://doi.org/10.1021/jf034974x

Brand, D., Pandey, a., Rodriguez-Leon, J. a., Roussos, S., Brand, I., & Soccol, C. R. (2001). Packed bed column fermenter and kinetic modeling for upgrading the nutritional quality of coffee husk in solid-state fermentation. *Biotechnology Progress*, *17*(6), 1065–1070. https://doi.org/10.1021/bp010112+

Bravo, J., Jua, I., Monente, C., Caemmerer, B., & Kroh, L. W. (2012). Evaluation of Spent Co ff ee Obtained from the Most Common Co ff eemakers as a Source of Hydrophilic Bioactive Compounds.

Bresciani, L., Calani, L., Bruni, R., Brighenti, F., & Del Rio, D. (2014). Phenolic composition, caffeine content and antioxidant capacity of coffee silverskin. *Food Research International*, *61*, 196–201. https://doi.org/10.1016/j.foodres.2013.10.047

Campos-Vega, R., Vázquez-Sánchez, K., López-Barrera, D., Loarca-Piña, G., Mendoza-Díaz, S., & Oomah, B. D. (2015). Simulated gastrointestinal digestion and in vitro colonic fermentation of spent coffee (Coffea arabica L.): Bioaccessibility and intestinal permeability. *Food Research International*, *77*, 156–161. https://doi.org/10.1016/j.foodres.2015.07.024

Campos-Vega, Rocio, Gaytan Martinez, M., Vazquez Sanchez, K., & Morales Sanchez, E. (2016). *MX/a/2016008578*.

Campos-Vega, Rocio, Loarca-Piña, G., Vergara-Castañeda, H., & Oomah, B. D. (2015). Spent coffee grounds: A review on current research and future prospects. *Trends in Food Science & Technology*, *45*(1), 24–36. https://doi.org/10.1016/j.tifs.2015.04.012

Chen, J. (2009). S.Café®. Retrieved from http://www.scafefabrics.com/en-global/home/index

Cherry, P. (1974). Method for drying moisture from wet spent coffee grounds.

Cho, A. S., Jeon, S. M., Kim, M. J., Yeo, J., Seo, K. Il, Choi, M. S., & Lee, M. K. (2010). Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. *Food and Chemical Toxicology*, *48*(3), 937–943.

https://doi.org/10.1016/j.fct.2010.01.003

Corrêa, J. L. G., Santos, J. C. P., Fonseca, B. E., & Carvalho, A. G. da S. (2014). Drying of spent coffee grounds in a cyclonic dryer. *Coffee Science*, *9*(1), 68–76. https://doi.org/10.25186/cs.v9i1.544

Costa, A. S. G., Alves, R. C., Vinha, A. F., Costa, E., Costa, C. S. G., Nunes, M. A., … Oliveira, M. B. P. P. (2018). Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product. *Food Chemistry*, *267*, 28–35. https://doi.org/10.1016/j.foodchem.2017.03.106

Costa, A. S. G. G., Alves, R. C., Vinha, A. F., Barreira, S. V. P. P., Nunes, M. a., Cunha, L. M., & Oliveira, M. B. P. P. P. (2014). Optimization of antioxidants extraction from coffee silverskin, a roasting by-product, having in view a sustainable process. *Industrial Crops and Products*, *53*, 350–357. https://doi.org/10.1016/j.indcrop.2014.01.006

Cubero-Castillo, E., Bonilla-Leiva, A. R., & García-Velasques, E. (2017). Coffee Berry Processing By-Product Valorization : Coffee Parchment as a Potential Fiber Source to Enrich Bakery Goods Abstract. *Journal of Food, Nutrition and Population Health*, *1*(2), 1–7.

De Bruyn, F., Zhang, S. J., Pothakos, V., Torres, J., Lambot, C., Moroni, A. V., … De Vuysta, L. (2017). Exploring the Impacts of Postharvest Processing on the Microbiota and Metabolite Profiles during Green Coffee Bean Production. *Applied and Environmental Microbiology*, *83*(1), 1–16.

del Castillo, M. Dolores, Fernandez-Gomez, B., Martinez-Saez, N., Iriondo-DeHond, A., & Mesa, M. D. (2019). Coffee By-Products. In A. Farah (Ed.), *Coffee: Production, Quality and Chemistry*. Oxfordshire: Royal Society of Chemistry.

del Castillo, Maria D., Iriondo-DeHond, A., Martinez-Saez, N., Fernandez-Gomez, B., Iriondo-DeHond, M., & Zhou, J.-R. (2017). Chapter 6 – Applications of recovered compounds in food products. In *Handbook of Coffee Processing By-Products* (pp. 171–194). https://doi.org/10.1016/B978-0-12-811290-8.00006-2

del Castillo, Maria Dolores, Fernandez-Gomez, B., Ullate, M., Mesa, M. D., & Del Castillo, M.D., Fernandez-Gomez, B., Ullate, M. and Mesa, M. D. (2016). *WO/2016/097450*. Oficina Española de Patentes y Marcas. Retrieved from https://patentscope.wipo.int/search/es/detail.jsf?docId=WO2016097450

del Castillo, María Dolores, Ibañez, M. E., Amigo, M., Herrero, M., Plaza del Moral, M., & Ullate, M. (2013). Application of products of coffee silverskin in anti-ageing cosmetics and functional food. WO 2013/004873.

del Castillo, María Dolores, Iriondo-DeHond, A., & Martirosyan, D. M. (2018). Are functional foods essential for sustainable health? *Annals of Nutrition & Food Science*.

del Castillo, María Dolores, Martinez-Saez, N., & Ullate, M. (2014). *PCT/ES2014/070062*. Spain.

Delzenne, N. M., Cani, P. D., Daubioul, C., & Neyrinck, A. M. (2005). Impact of inulin and oligofructose on gastrointestinal peptides. *British Journal of Nutrition*, *93*(S1), S157.

https://doi.org/10.1079/BJN20041342

Demir, M. K., & Elgün, A. (2014). Comparison of autoclave, microwave, IR and UV-C stabilization of whole wheat flour branny fractions upon the nutritional properties of whole wheat bread. *Journal of Food Science and Technology*, *51*(1), 59–66. https://doi.org/10.1007/s13197-011-0475-0

Deora, N. S. (2018). Whole wheat flour stability: An insight. *Acta Scientific Nutritioal Health*, *2*(3), 8–18.

Diaz, V. C. (2019). Know the Novel Foods. Retrieved from https://knowmadinstitut.org/2019/05/know-the-novel-foods/

Du, H., van der A, D. L., Boshuizen, H. C., Forouhi, N. G., Wareham, N. J., Halkjær, J., … Feskens, E. J. (2010). Dietary fiber and subsequent changes in body weight and waist circumference in European men and women. *The American Journal of Clinical Nutrition*, *91*(2), 329–336. https://doi.org/10.3945/ajcn.2009.28191

EFSA Panel on Dietetic Products. (2016). Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. *EFSA Journal Journal*, *14*(September), 11. https://doi.org/10.2903/j.efsa.2016.NNNN

Esquivel, P., & Jiménez, V. M. (2012). Functional properties of coffee and coffee by-products. *Food Research International*, *46*(2), 488–495. https://doi.org/10.1016/j.foodres.2011.05.028

European Comission. (2019). Novel food catalogue.

European Commission. (2005). COMMISSION REGULATION (EC) No 123/2005 of 26 January 2005 amending Regulation (EC) No 466/2001 as regards ochratoxin A. *Official Journal of the European Union*, *2005*(123), 2004–2006. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R0123&qid=1478028660364&from=ES

European Commission. (2012). COMMISSION REGULATION (EU) No 1047/2012. *Official Journal of the European Union*, *2012*(1047), 36–37.

European Commission. (2017). COMMISSION REGULATION (EU) 2017/2158 of 20 November 2017 establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food. *Official Journal of the European Union*, *204*, 24–44. https://doi.org/http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_285/l_28520031101en00330037.pdf

European Commission. (2018). Bioeconomy, FOOD 2030. Retrieved from https://ec.europa.eu/research/bioeconomy/index.cfm?pg=policy&lib=food2030

European Council. (1990). Council Directive of 24 September 1990 on nutrition labelling for foodstuffs (90/496/ EEC). *Official Journal of the European Union*, (L), 18–63. Retrieved from http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32011R1169

European Food Safety Authority. (2015). Scientific Opinion on acrylamide in food. *EFSA Journal*, *13*(6), 4104. https://doi.org/10.2903/j.efsa.2015.4104

European Food Safety Authority (EFSA). (2010). Scientific Opinion on Dietary Reference Values for carbohydrates and dietary fibre. *EFSA Journal*, *8*(3), 1–77. https://doi.org/10.2903/j.efsa.2010.1462.Available

European Food Safety Authority (EFSA). (2015). Scientific Opinion on the safety of caffeine. *EFSA Journal*, *13*(5), 1–21. https://doi.org/10.2903/j.efsa.2015.4102

Fahim, I. S., Chbib, H., & Mahmoud, H. M. (2019). The synthesis, production & economic feasibility of manufacturing PLA from agricultural waste. *Sustainable Chemistry and Pharmacy*, *12*(March), 100142. https://doi.org/10.1016/j.scp.2019.100142

Fairweather, A. (n.d.). Green Cup. Retrieved from http://greencup.co.uk/recycling-upcycling/

FAO, & Food Climate Research Network. (2016). *Plates, pyramids, planet*. https://doi.org/ISBN 978-92-5-109222-4

Farah, A. (2012). Coffee Constituents. *Coffee: Emerging Health Effects and Disease Prevention*, 21–58. https://doi.org/10.1002/9781119949893.ch2

Fernández-Gómez, B. (2016). Sustainable use of coffee silverskin as a natural source of bioactive compounds for diabetes, (April), 87–132.

Fernandez-Gomez, B., Lezama, A., Amigo-Benavent, M., Ullate, M. M., Herrero, M., Martín, M. Á., … del Castillo, M. D. M. D. (2016). Insights on the health benefits of the bioactive compounds of coffee silverskin extract. *Journal of Functional Foods*, *25*(6), 197–207. https://doi.org/10.1016/j.jff.2016.06.001

Fernandez-Gomez, B., Ramos, S., Goya, L., Mesa, M. D., del Castillo, M. D., & Martín, M. Á. (2016). Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells. *Food Research International*. https://doi.org/.doi.org/10.1016/j.foodres.2016.03.006

Fernandez-Gomez, B., Ullate, M., Picariello, G., Ferranti, P., Mesa, M. D., & del Castillo, M. D. (2015). New knowledge on the antiglycoxidative mechanism of chlorogenic acid. *Food Funct.*, *6*(6), 2081–2090. https://doi.org/10.1039/C5FO00194C

Fernandez-Vasallo, C. (2017). *Harinas especiales*. *XXIX AETC Jornadas Técnicas*. Valladolid. Retrieved from http://www.aetc.es/wp-content/uploads/2017/11/Harinas-Especiales.-Carlos-Fernández-Vasallo.-MOLENDUM-1.pdf

Ferrão, J. E. M. (2009). *O Café, A Bebida Negra Dos Sonhos Claros*. Lisboa, Portugal.: Chaves Ferreira-Publicações S. A.

Ferraz, M. B. M., Farah, A., Iamanaka, B. T., Perrone, D., Copetti, M. V., Marques, V. X., … Taniwaki, M. H. (2010). Kinetics of ochratoxin A destruction during coffee roasting. *Food Control*, *21*(6), 872–877. https://doi.org/10.1016/j.foodcont.2009.12.001

Food and Agriculture Organization of the United Nations (FAO). (2011). *Global food losses and food waste – Extent, causes and prevention*. Rome. https://doi.org/10.1098/rstb.2010.0126

Food and Agriculture Organization of the United Nations (FAO). (2018). Sustainable Bioeconomy Guidelines. Retrieved from http://www.fao.org/energy/bioeconomy/en/

Food and Agriculture Organization of the United Nations (FAO). (2019). Development of a Code of Conduct on Food Loss and Food Waste Prevention. Retrieved from http://www.fao.org/fsnforum/es/node/4877

Furusawa, M., Narita, Y., Iwai, K., Fukunaga, T., & Nakagiri, O. (2011). Inhibitory effect of a hot water extract of coffee "silverskin" on hyaluronidase. *Bioscience, Biotechnology, and Biochemistry*, *75*(6), 1205–1207. https://doi.org/10.1271/bbb.110106

FUTURE CEUTICALS. (1999). Retrieved from https://www.futureceuticals.com/

Garcia-Serna, E., Martinez-Saez, N., Mesias, M., Morales, F. J., & Castillo, M. D. Del. (2014). Use of Coffee Silverskin and Stevia to Improve the Formulation of Biscuits. *Polish Journal of Food and Nutrition Sciences*, *64*(4), 243–251. https://doi.org/10.2478/pjfns-2013-0024

Gómez-De La Cruz, F. J., Cruz-Peragón, F., Casanova-Peláez, P. J., & Palomar-Carnicero, J. M. (2015). A vital stage in the large-scale production of biofuels from spent coffee grounds: The drying kinetics. *Fuel Processing Technology*, *130*(C), 188–196. https://doi.org/10.1016/j.fuproc.2014.10.012

Gonzalez, B. (2016). *Uso sostenible del pergamino de café como ingrediente alimentario en la elaboración de pan libre de gluten con alto contenido en fibra*. Universidad Autónoma de Madrid.

Gouvea, B. M., Torres, C., Franca, A. S., Oliveira, L. S., & Oliveira, E. S. (2009). Feasibility of ethanol production from coffee husks. *Biotechnology Letters*, *31*(9), 1315–1319. https://doi.org/10.1007/s10529-009-0023-4

Guglielmetti, A., Fernandez-Gomez, B., Zeppa, G., & del Castillo, M. D. (2019). Nutritional quality, potential health promoting properties and sensory perception of an improved gluten free bread formulation containing inulin, rice protein and bioactive compounds extracted from coffee byproducts. *Polish Journal of Food and Nutrition Sciences*, *Accepted*. https://doi.org/10.31883/pjfns-2019-0012

Hartati, I., Wahid, U., Riwayati, I., Wahid, U., Kurniasari, L., Wahid, U., & Hartati, I. (2012). Potential production of food colorant from coffee pulp, (December 2015).

Heeger, A., Kosińska-Cagnazzo, A., Cantergiani, E., & Andlauer, W. (2017). Bioactives of coffee cherry pulp and its utilisation for production of Cascara beverage. *Food Chemistry*, *221*, 969–975. https://doi.org/10.1016/j.foodchem.2016.11.067

Heimbach, J. T., Marone, P. A., Hunter, J. M., Nemzer, B. V., Stanley, S. M., & Kennepohl, E. (2010). Safety studies on products from whole coffee fruit. *Food and Chemical Toxicology*, *48*(8–9), 2517–2525. https://doi.org/10.1016/j.fct.2010.06.025

Hwang, J. H., Kim, K. J., Ryu, S. J., & Lee, B. Y. (2016). Caffeine prevents LPS-induced inflammatory responses in RAW264.7 cells and zebrafish. *Chemico-Biological Interactions*, *248*, 1–7. https://doi.org/10.1016/j.cbi.2016.01.020

Hwang, S. J., Kim, Y. W., Park, Y., Lee, H. J., & Kim, K. W. (2014). Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide- stimulated RAW 264.7 cells. *Inflammation Research*, *63*(1), 81–90. https://doi.org/10.1007/s00011-013-0674-4

Imbert, E. (2017). Food waste valorization options: opportunities from the bioeconomy. *Open*

Agriculture, *2*(1), 195–204. https://doi.org/10.1515/opag-2017-0020

International Coffee Organization. (2019). *Total production by all exporting countries*. *Trade Statistics Tables*.

Iriondo-DeHond, M. (2019). *Development of novel functional dairy foods using byproducts from the coffee and wine industries for a sustainable nutrition and health*. Universidad Autonóma de Madrid.

Janissen, B., & Huynh, T. (2018). Chemical composition and value-adding applications of coffee industry by-products: A review. *Resources, Conservation and Recycling*, *128*(July 2017), 110–117. https://doi.org/10.1016/j.resconrec.2017.10.001

Jelderks, J., & Zimmermann, U. (2017). Caskai. Retrieved from https://caskai.com/

Jiménez-Zamora, A., Pastoriza, S., & Rufián-Henares, J. a. (2015). Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties. *LWT - Food Science and Technology*, *61*(1), 12–18. https://doi.org/10.1016/j.lwt.2014.11.031

K., K., L., P., & V.P., M. (2010). Combined treatment of tetrahydrocurcumin and chlorogenic acid exerts potential antihyperglycemic effect on streptozotocin-nicotinamide-induced diabetic rats. *General Physiology and Biophysics*, *29*(1), 23–30. https://doi.org/10.4149/gpb_2010_01_23

KAFFEE FORM. (2015). Retrieved September 19, 2019, from https://www.kaffeeform.com/de/

Kamal, I. M., Sobolik, V., Kristiawan, M., Mounir, S. M., & Allaf, K. (2008). Structure expansion of green coffee beans using instantaneous controlled pressure drop process. *Innovative Food Science and Emerging Technologies*, *9*(4), 534–541. https://doi.org/10.1016/j.ifset.2008.01.004

Kobayashi-Hattori, K., Mogi, A., Matsumoto, Y., & Takita, T. (2005). Effect of caffeine on the body fat and lipid metabolism of rats fed on a high-fat diet. *Biosci Biotechnol Biochem*, *69*(11), 2219–2223. https://doi.org/10.1271/bbb.69.2219

Kumar, K., Yadav, A. N., Kumar, V., Vyas, P., & Dhaliwal, H. S. (2017). Food waste: a potential bioresource for extraction of nutraceuticals and bioactive compounds. *Bioresources and Bioprocessing*, *4*(1), 18. https://doi.org/10.1186/s40643-017-0148-6

Limousy, L., Jeguirim, M., Labbe, S., Balay, F., & Fossard, E. (2015). Performance and emissions characteristics of compressed spent coffee ground/wood chip logs in a residential stove. *Energy for Sustainable Development*, *28*, 52–59. https://doi.org/10.1016/J.ESD.2015.07.002

López-Barrera, D. M., Vázquez-Sánchez, K., Loarca-Piña, M. G. F., & Campos-Vega, R. (2016). Spent coffee grounds, an innovative source of colonic fermentable compounds, inhibit inflammatory mediators in vitro. *Food Chemistry*, *212*, 282–290. https://doi.org/10.1016/j.foodchem.2016.05.175

Louis Bresson, J., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., Ildico Hirsch-Ernst, K., … Vinceti, M. (2016). SCIENTIFIC OPINION Draft guidance on the preparation and presentation of an application for authorisation of a Novel Food EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) Novel Food Guidance Panel members.

EFSA Journal Journal. https://doi.org/10.2903/j.efsa.2016.NNNN

Magoni, C., Bruni, I., Guzzetti, L., Dell'Agli, M., Sangiovanni, E., Piazza, S., … Labra, M. (2018). Valorizing coffee pulp by-products as anti-inflammatory ingredient of food supplements acting on IL-8 release. *Food Research International*, *112*, 129–135. https://doi.org/10.1016/J.FOODRES.2018.06.026

Martinez-Saez, N., Hochkogler, C., Somoza, V., & del Castillo, M. (2017). Biscuits with No Added Sugar Containing Stevia, Coffee Fibre and Fructooligosaccharides Modifies α-Glucosidase Activity and the Release of GLP-1 from HuTu-80 Cells and Serotonin from Caco-2 Cells after In Vitro Digestion. *Nutrients*, *9*(7), 694. https://doi.org/10.3390/nu9070694

Martinez-Saez, N., Tamargo García, A., Domínguez Pérez, I., Rebollo-Hernanz, M., Mesías, M., Morales, F. J., … del Castillo, M. D. (2017). Use of spent coffee grounds as food ingredient in bakery products. *Food Chemistry*, *216*, 114–122. https://doi.org/10.1016/j.foodchem.2016.07.173

Martinez-Saez, N., Ullate, M., Martin-Cabrejas, M. a., Martorell, P., Genovés, S., Ramon, D., & Del Castillo, M. D. (2014). A novel antioxidant beverage for body weight control based on coffee silverskin. *Food Chemistry*, *150*, 227–234. https://doi.org/10.1016/j.foodchem.2013.10.100

Marto, J., Gouveia, L. F., Chiari, B. G., Paiva, A., Isaac, V., Pinto, P., … Ribeiro, H. M. (2016). The green generation of sunscreens: Using coffee industrial sub-products. *Industrial Crops and Products*, *80*, 93–100. https://doi.org/10.1016/J.INDCROP.2015.11.033

Massachusetts Institute of Technology. (2010). Ministry of Supply. Retrieved from https://ministryofsupply.com/

Mata, T. M., Martins, A. A., & Caetano, N. S. (2018). Bio-refinery approach for spent coffee grounds valorization. *Bioresource Technology*, *247*(July 2017), 1077–1084. https://doi.org/10.1016/j.biortech.2017.09.106

McMillan, R. (2014). ROSALIE MCMILLAN. Retrieved from http://www.rosaliemcmillan.com/

McNutt, J., & He, Q. (Sophia). (2019). Spent coffee grounds: A review on current utilization. *Journal of Industrial and Engineering Chemistry*, *71*, 78–88. https://doi.org/10.1016/j.jiec.2018.11.054

Meijer, K., De Vos, P., & Priebe, M. G. (2010). Butyrate and other short-chain fatty acids as modulators of immunity: What relevance for health? *Current Opinion in Clinical Nutrition and Metabolic Care*, *13*(6), 715–721. https://doi.org/10.1097/MCO.0b013e32833eebe5

Mesías, M., & Delgado-Andrade, C. (2017). Melanoidins as a potential functional food ingredient. *Current Opinion in Food Science*, *14*, 37–42. https://doi.org/10.1016/j.cofs.2017.01.007

Meyer, K. A., Kushi, L. H., Jacobs, D. R., Slavin, J., Sellers, T. A., & Folsom, A. R. (2000). Carbohydrates, dietary fiber, and incident type 2 diabetes in older women. *The American Journal of Clinical Nutrition*, *71*(4), 921–930. https://doi.org/10.1093/ajcn/71.4.921

Michalska, A., Honke, J., Łysiak, G., & Andlauer, W. (2016). Effect of drying parameters on the formation of early and intermediate stage products of the Maillard reaction in different plum (Prunus domestica L.) cultivars. *LWT - Food Science and Technology*, *65*, 932–938. https://doi.org/10.1016/j.lwt.2015.09.015

Miljkovic, D. (2011). Coffee cherry cosmetic composition and methods. *Dusan Miljkovic*.

Moreira, A. S. P., Nunes, F. M., Domingues, M. R., & Coimbra, M. A. (2012). Coffee melanoidins: structures, mechanisms of formation and potential health impacts. *Food & Function*, *3*(9), 903. https://doi.org/10.1039/c2fo30048f

Moreira, A. S. P., Nunes, F. M., Simões, C., Maciel, E., Domingues, P., Domingues, M. R. M., & Coimbra, M. A. (2017). Transglycosylation reactions, a main mechanism of phenolics incorporation in coffee melanoidins: Inhibition by Maillard reaction. *Food Chemistry*, *227*, 422–431. https://doi.org/10.1016/j.foodchem.2017.01.107

Murthy, P. S., & Madhava Naidu, M. (2012). Sustainable management of coffee industry by-products and value addition—A review. *Resources, Conservation and Recycling*, *66*, 45–58. https://doi.org/10.1016/j.resconrec.2012.06.005

Murthy, P. S., Manjunatha, M. R., Sulochannama, G., Madhava Naidu, M., Pushpa S. Murthy, M.R. Manjunatha, G. S. and M. M. N., Murthy, P. S., … Naidu, M. M. (2012). Extraction, Characterization and Bioactivity of Coffee Anthocyanins. *European Journal of Biological Sciences*, *4*(1), 13–19. https://doi.org/10.5829/idosi.ejbs.2012.4.1.6149

Murthy, P. S., & Naidu, M. M. (2012). Recovery of Phenolic Antioxidants and Functional Compounds from Coffee Industry By-Products. *Food and Bioprocess Technology*, *5*(3), 897–903. https://doi.org/10.1007/s11947-010-0363-z

Mussatto, S. I., Carneiro, L. M., Silva, J. P. A., Roberto, I. C., & Teixeira, J. A. (2011). A study on chemical constituents and sugars extraction from spent coffee grounds. *Carbohydrate Polymers*, *83*(2), 368–374. https://doi.org/10.1016/j.carbpol.2010.07.063

Mussatto, S. I., Machado, E. M. S., Martins, S., & Teixeira, J. a. (2011). Production, Composition, and Application of Coffee and Its Industrial Residues. *Food and Bioprocess Technology*, *4*(5), 661–672. https://doi.org/10.1007/s11947-011-0565-z

Napolitano, A., Fogliano, V., Tafuri, A., & Ritieni, A. (2007). Natural occurrence of ochratoxin A and antioxidant activities of green and roasted coffees and corresponding byproducts. *Journal of Agricultural and Food Chemistry*, *55*(25), 10499–10504. https://doi.org/10.1021/jf071959+

Narita, Y., & Inouye, K. (2014). Review on utilization and composition of coffee silverskin. *Food Research International*, *61*, 16–22. https://doi.org/10.1016/j.foodres.2014.01.023

Natucafé S.A.S. (2019). Coffee Mucilage Concentrate. Retrieved from http://natucafe.co/coffee-mucilage-concentrate.php

Oliveira, L. S., & Franca, A. S. (2014). *An Overview of the Potential Uses for Coffee Husks*. *Coffee in Health and Disease Prevention*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-409517-5.00031-0

Orrego, D., Zapata-Zapata, A. D., & Kim, D. (2018). Optimization and scale-up of coffee

mucilage fermentation for ethanol production. *Energies*, *11*(4), 1–12. https://doi.org/10.3390/en11040786

Pandey, A., Soccol, C. R., Nigam, P., Brand, D., Mohan, R., & Roussos, S. (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochemical Engineering Journal*, *6*(2), 153–162. https://doi.org/10.1016/S1369-703X(00)00084-X

Pearson, K. (2015). Nutraceuticals and skin health: what are the key benefits and protective properties? *Journal of Aesthetic Nursing*, *4*(4), 166–171. https://doi.org/10.12968/joan.2015.4.4.166

Pectcof B.V. (2019). Retrieved from https://pectcof.com/

Pedreschi, F., Mariotti, M. S., & Granby, K. (2014). Current issues in dietary acrylamide: Formation, mitigation and risk assessment. *Journal of the Science of Food and Agriculture*, *94*(1), 9–20. https://doi.org/10.1002/jsfa.6349

Porres, C., Alvarez, D., & Calzada, J. (1993). Caffeine reduction in coffee pulp through silage. *Biotechnology Advances*, *11*(3), 519–523. https://doi.org/10.1016/0734-9750(93)90020-N

Pourfarzad, A., Mahdavian-Mehr, H., & Sedaghat, N. (2013). Coffee silverskin as a source of dietary fiber in bread-making: Optimization of chemical treatment using response surface methodology. *LWT - Food Science and Technology*, *50*(2), 599–606. https://doi.org/10.1016/j.lwt.2012.08.001

Prata, E. R. B. A. B. a, & Oliveira, L. S. (2007). Fresh coffee husks as potential sources of anthocyanins. *LWT - Food Science and Technology*, *40*(9), 1555–1560. https://doi.org/10.1016/j.lwt.2006.10.003

Ramalakshmi, K., Rao, L. J. M., Takano-Ishikawa, Y., & Goto, M. (2009). Bioactivities of low-grade green coffee and spent coffee in different in vitro model systems. *Food Chemistry*, *115*(1), 79–85. https://doi.org/10.1016/j.foodchem.2008.11.063

Ramirez-Coronel, M. A., Marnet, N., Kolli, V. S. K., Roussos, S., Guyot, S., & Augur, C. (2004). Characterization and estimation of proanthocyanidins and other phenolics in coffee pulp (Coffea arabica) by thiolysis-high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, *52*(5), 1344–1349. https://doi.org/10.1021/jf035208t

Ramirez-Martinez, J. R. (1988). Phenolic compounds in coffee pulp: Quantitative determination by HPLC. *Journal of the Science of Food and Agriculture*, *43*(2), 135–144. https://doi.org/10.1002/jsfa.2740430204

Ramirez Velez, A., & Jaramillo Lopez, J. C. (2015). Process for obtaining honey and/or flour of coffee from the pulp or husk and the mucilage of the coffee bean. US20150017270 A1. Colombia.

Rathinavelu, R., & Graziosi, G. (2005). Potential alternative use of coffee wastes and by-products, (942), 1–4.

Rebollo-Hernanz, M., Zhang, Q., Aguilera, Y., Martín-Cabrejas, M. A., & Gonzalez de Mejia, E. (2019). Phenolic compounds from coffee by-products modulate adipogenesis-related inflammation, mitochondrial dysfunction, and insulin resistance in adipocytes, via insulin/PI3K/AKT signaling pathways. *Food and Chemical Toxicology*, *132*, 110672.

https://doi.org/10.1016/j.fct.2019.110672

Rios, M. B. (2018). *Application of coffee cascara as a source of dietary fiber in the preparation of gluten-free breads*. Universidad Politécnica de Madrid.

Rocha, M. V. P., de Matos, L. J. B. L., Lima, L. P. De, Figueiredo, P. M. D. S., Lucena, I. L., Fernandes, F. A. N., & Gonçalves, L. R. B. (2014). Ultrasound-assisted production of biodiesel and ethanol from spent coffee grounds. *Bioresource Technology*, *167*, 343–348. https://doi.org/10.1016/j.biortech.2014.06.032

Rodrigues, F., Pereira, C., Pimentel, F. B., Alves, R. C., Ferreira, M., Sarmento, B., … Oliveira, M. B. P. P. (2015). Are coffee silverskin extracts safe for topical use? An in vitro and in vivo approach. *Industrial Crops and Products*, *63*, 167–174. https://doi.org/10.1016/j.indcrop.2014.10.014

Rodrigues, F, Sarmento, B., Amaral, M. H., & Oliveira, M. B. P. P. (2016). Exploring the antioxidant potentiality of two food by-products into a topical cream: stability, in vitro and in vivo evaluation. *Drug Development and Industrial Pharmacy*, *42*(6), 880–889. https://doi.org/10.3109/03639045.2015.1088865

Rodrigues, Francisca, Matias, R., Ferreira, M., Amaral, M. H., & Oliveira, M. B. P. P. (2016). In vitro and in vivo comparative study of cosmetic ingredients Coffee silverskin and hyaluronic acid. *Experimental Dermatology*, *25*(7), 572–574. https://doi.org/10.1111/exd.13010

Ronga, D., Pane, C., Zaccardelli, M., & Pecchioni, N. (2016). Use of Spent Coffee Ground Compost in Peat-Based Growing Media for the Production of Basil and Tomato Potting Plants. *Communications in Soil Science and Plant Analysis*, *47*(3), 356–368. https://doi.org/10.1080/00103624.2015.1122803

Sahasrabudhe, A., & Deodhar, M. (2010). Anti-hyaluronidase, anti-elastase activity of Garcinia indica. *International Journal of Botany*. https://doi.org/10.3923/ijb.2010.299.303

Sampaio, A., Dragone, G., Vilanova, M., Oliveira, J. M., Teixeira, J. a., & Mussatto, S. I. (2013). Production, chemical characterization, and sensory profile of a novel spirit elaborated from spent coffee ground. *LWT - Food Science and Technology*, *54*(2), 557–563. https://doi.org/10.1016/j.lwt.2013.05.042

Secret Cherry Tea. (2019). Superfood Coffee Cherry Tea. Retrieved from https://www.secretcherrytea.com/

Seo, J., Jung, J. K., & Seo, S. (2015). Evaluation of nutritional and economic feed values of spent coffee grounds and *Artemisia princeps* residues as a ruminant feed using *in vitro* ruminal fermentation. *PeerJ*, *3*, e1343. https://doi.org/10.7717/peerj.1343

Sleeth, M. L., Thompson, E. L., Ford, H. E., Zac-Varghese, S. E. K., & Frost, G. (2010). Free fatty acid receptor 2 and nutrient sensing: A proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation. *Nutrition Research Reviews*, *23*(1), 135–145. https://doi.org/10.1017/S0954422410000089

St-Pierre, D. H., Rabasa-Lhoret, R., Lavoie, M. È., Karelis, A. D., Strychar, I., Doucet, E., & Coderre, L. (2009). Fiber intake predicts ghrelin levels in overweight and obese

postmenopausal women. *European Journal of Endocrinology*, *161*(1), 65–72. https://doi.org/10.1530/EJE-09-0018

Tarini, J., & Wolever, T. M. S. (2010). The fermentable fibre inulin increases postprandial serum short-chain fatty acids and reduces free-fatty acids and ghrelin in healthy subjects. *Applied Physiology, Nutrition, and Metabolism*, *35*(1), 9–16. https://doi.org/10.1139/H09-119

The Coffee Cherry Co. (2014). Retrieved from https://coffeecherryco.com/

The European Commission. (2012). Commission Regulation (EU) No 432/2012. *Official Journal of the European Union*, *13*(1924). Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32012R0432&from=EN

The European Parliament and The Council of the European Union. (2006). REGULATION (EC) No 1924/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on nutrition and health claims made on foods. *Official Journal of the European Union*, (404), 9–25. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1924&from=en

The European Parliament and The Council of the European Union. (2015). Regulation (Eu) 2015/2283 on novel foods. *Official Journal of the European Union*, *327*(258), 1–22.

Torres-León, C., Ramírez-Guzman, N., Londoño-Hernandez, L., Martinez-Medina, G. A., Díaz-Herrera, R., Navarro-Macias, V., … Aguilar, C. N. (2018). Food Waste and Byproducts: An Opportunity to Minimize Malnutrition and Hunger in Developing Countries. *Frontiers in Sustainable Food Systems*, *2*(September), 1–17. https://doi.org/10.3389/fsufs.2018.00052

Toschi, T. G., Cardenia, V., Bonaga, G., Mandrioli, M., & Rodriguez-estrada, M. T. (2014). Coffee Silverskin : Characterization , Possible Uses , and Safety Aspects. *Journal of Agricultural and Food Chemistry*, *62*, 10836–10844.

US Department of Agriculture. (2015). *Scientific Report of the 2015 Dietary Guidelines Advisory Committee*. Retrieved from https://health.gov/dietaryguidelines/2015-scientific-report/pdfs/scientific-report-of-the-2015-dietary-guidelines-advisory-committee.pdf

USDA Foreign Agricultural Service. (2019). Coffee: World Markets and Trade. *Coffee: World Markets and Trade*. Retrieved from http://apps.fas.usda.gov/psdonline/circulars/coffee.pdf

Vazquez Sanchez, K., Martinez-Saez, N., del Castillo, M. D., & Campos-Vega, R. (2015). Antioxidant coffee dietary fiber for gastrointestinal health and diabetes. In D. M. Martirosyan (Ed.), *Functional and medical foods for chronic diseases: bioactive compounds and biomarkers*. The 20th International Conference, Boston, USA. 22-23 September, 2016.

Vilela, W. F., Leão, D. P., Franca, A. S., & Oliveira, L. S. (2016). Effect of Peroxide Treatment on Functional and Technological Properties of Fiber-Rich Powders Based on Spent Coffee Grounds. *International Journal of Food Engineering*, *2*(1). https://doi.org/10.18178/ijfe.2.1.42-47

WHO. (2003). Diet, nutrition and the prevention of chronic diseases. *World Health Organization Technical Report Series*, *916*, i-viii-1-149-backcover. https://doi.org/ISBN 92 4 120916 X

ISSN 0512-3054 (NLM classification: QU 145)

Williamson, K., & Hatzakis, E. (2019). NMR analysis of roasted coffee lipids and development of a spent ground coffee application for the production of bioplastic precursors. *Food Research International*, *119*, 683–692. https://doi.org/10.1016/J.FOODRES.2018.10.046

World Health Organization (WHO). (2015). European Food and Nutrition Action Plan 2015 - 2020.

Yang, J., Ching, Y. C., & Chuah, C. H. (2019). Applications of lignocellulosic fibers and lignin in bioplastics: A review. *Polymers*, *11*(5), 1–26. https://doi.org/10.3390/polym11050751

Zhang, X., Tao, N., Wang, X., Chen, F., & Wang, M. (2015). The colorants, antioxidants, and toxicants from nonenzymatic browning reactions and the impacts of dietary polyphenols on their thermal formation. *Food and Function*, *6*(2), 345–355. https://doi.org/10.1039/c4fo00996g

CONCLUSIONS/ CONCLUSIONES

CONCLUSIONS

Results derived from the present PhD thesis led to the following conclusions:

1. Coffee husk can be converted into two novel safe food ingredients containing nutrients and non-nutrient bioactive compounds. The fractioning procedure is a sustainable, simple, green and easy to scale-up process by the coffee industry achieving a whole waste recovery and avoiding new investment. An enriched extract with multifunctional properties and an insoluble fraction composed mainly by dietary fiber with different structure and function (soluble and insoluble) were obtained.

2. Coffee parchment is mainly composed (92 %) by insoluble dietary fiber such as cellulose and lignin. It can be used as a single food ingredient being a natural, safe and sustainable source of antioxidant dietary fiber after a sole stabilization/sanitation process. The use of this by-product for plastic replacement in the food industry is also feasible and of great interest.

3. Coffee silverskin can be repurposed in two safe and sustainable ingredients: an aqueous extract (CSE) composed by nutrients and non-nutrients with health promoting properties and an insoluble fraction enriched in dietary fiber (soluble and insoluble). Full recovery of the by-product as food ingredients represents an important advantage against its incineration, which is a common practice.

4. CSE safety was proved by genotoxicity, cytotoxicity, acute (2 g/kg b.w.) and repeated dose (1 g/kg b.w.) toxicological studies. The extract has particular beneficial nutritional properties, consequently it can be repurposed as a novel food ingredient with the following nutrition claims: source of proteins, potassium, magnesium, calcium and vitamin C, low in fat and high in fiber. It resulted as a chemopreventive agent against benzo (a) pyrene. The supplementation of the diet of healthy animals with CSE did not affect key physiological functions (hormone secretion, antioxidant status and anti-inflammatory biomarkers). Its dietary fiber was fermented *in vivo* giving rise to short chain fatty acids (SCFAs).

5. Instant spent coffee grounds (SCGs) safety was validated by mycotoxins analysis, acute and repeated treatments of Wistar rats. Fiber and fat are the major components of SCGs. They acutely accelerated intestinal motility in rats. Most of the saturated fatty acids and diterpenes (cafestol and kahweol) composing SCGs were not

bioaccesible after *in vitro* digestion. Therefore, SCGs can be considered a sustainable, safe and healthy food ingredient with potential for preventing hepatic steatosis due to their effect as dietary fiber with a high fat-holding capacity.

6. Melanoidins, isolated biomolecules from CSE and SCGs (>10 kDa), showed antioxidant properties *in vitro* and in human colon cell lines. They might be defined as "Maillardized antioxidant dietary fiber". Isolated CSE fraction enriched in melanoidins (1 g/kg b.w.) accelerated intestinal transit in rats.

7. Prior to the routine and large-scale development of food ingredients, coffee by-products (husk, parchment, silverskin and spent coffee grounds) must be certified for food safety by the analysis of chemical (pesticides), biological (microorganisms and mycotoxins) and processing contaminants (acrylamide).

In summary, the present PhD thesis showed the feasibility of using coffee by-products as sustainable and safe novel ingredients composed by nutrients and non-nutrient bioactive compounds. Enough scientific basis for the validation and authorization by the regulatory bodies of husk, CSE and SCGs as Novel Foods has been obtained during this investigation. The use of coffee by-products as novel ingredients contributes to the sustainability of the coffee sector, to the reduction its negative environmental impact, to an efficient use of natural resources and to achieve a sustainable nutrition and health for global population.

CONCLUSIONES

Los resultados derivados de la presente tesis doctoral llevaron a las siguientes conclusiones:

1. La cáscara de café se fraccionó en dos nuevos ingredientes alimentarios seguros, sostenibles y compuestos por nutrientes y no-nutrientes bioactivos. El proceso para su fraccionamiento es sostenible, simple, ecológico y fácil de escalar a nivel industrial, logrando una recuperación total del subproducto y sin necesidad de nuevas inversiones para el sector cafetero. Se obtuvo un extracto enriquecido con propiedades multifuncionales y una fracción insoluble compuesta principalmente por fibra dietética con diferente estructura y función (soluble e insoluble).

2. El pergamino, tras un único paso de estabilización/higienización, se propone como un único ingrediente alimentario fuente natural, segura y sostenible de fibra dietética antioxidante. Este está compuesto principalmente (92 %) por fibra dietética insoluble tales como la celulosa y la lignina. El uso de este subproducto para la sustitución del plástico en la industria alimentaria también es factible y de gran interés.

3. La cascarilla de café se reconvirtió en dos ingredientes alimentarios seguros y sostenibles: un extracto acuoso compuesto por nutrientes y no-nutrientes con propiedades promotoras de la salud y una fracción insoluble enriquecida en fibra dietética (soluble e insoluble). La recuperación total del subproducto como ingredientes alimentarios representa una ventaja importante frente a su incineración, que es la práctica habitual.

4. El extracto acuoso de cascarilla de café (CSE, sus siglas en inglés) es seguro como ingrediente alimentario. Los resultados de genotoxicidad, citotoxicidad, toxicidad aguda (2 g/kg p.c.) y toxicidad de dosis repetidas (1 g/kg p.c.) de la presente memoria avalan este hecho. El extracto tiene una composición nutricional particular y se le pueden asignar las alegaciones: fuente de proteínas, potasio, magnesio, calcio y vitamina C, bajo en grasas y alto en fibra. CSE presenta potencial quimiopreventivo contra el benzo (a) pireno. La suplementación de la dieta de animales sanos con este extracto no afecta funciones fisiológicas clave (secreción hormonal, estado antioxidante y biomarcadores antiinflamatorios). La fibra dietética presente en CSE se fermenta *in vivo* dando lugar a ácidos grasos de cadena corta (SCFAs, sus siglas en inglés).

5. Los posos del proceso de obtención del café instantáneo (SCGs, sus siglas en inglés) son seguros como ingrediente alimentario atendiendo a los resultados obtenidos mediante el análisis de micotoxinas, y estudios de toxicidad aguda y de dosis repetidas en ratas Wistar. La fibra y la grasa son los principales componentes de los posos. La ingesta de una única dosis de SCGs (1 g/kg p.c.) acelera la motilidad intestinal en ratas. La mayor parte de los ácidos grasos saturados y diterpenos (cafestol y kahweol) que componen los SCGs no están bioaccesibles después de la digestión *in vitro*. Por tanto, los SCGs pueden considerarse como un ingrediente alimentario sostenible, seguro y saludable con potencial para prevenir la esteatosis hepática debido a su efecto como fibra dietética con una alta capacidad de retención de grasa.

6. Las melanoidinas, biomoléculas aisladas de CSE y SCGs (> 10 kDa), presentan propiedades antioxidantes *in vitro* y en líneas celulares de colon humano. Se pueden definir como "fibra dietética antioxidante Maillardizada". La fracción aislada de CSE enriquecida en melanoidinas (1 g/kg p.c.) acelera el tránsito intestinal en ratas.

7. La materia prima previo a la obtención rutinaria y a gran escala de ingredientes alimentarios a partir de subproductos del café (cáscara, pergamino, cascarilla y posos) debe ser certificada para garantizar su seguridad alimentaria mediante análisis de contaminantes químicos (pesticidas), biológicos (microorganismos y micotoxinas) y del procesado (acrilamida).

En conclusión, la presente tesis doctoral muestra la viabilidad de los subproductos del café como nuevos ingredientes sostenibles, seguros y compuestos por nutrientes y no-nutrientes bioactivos. La presente investigación aporta la base científica suficiente y necesaria para la validación y autorización por parte de los organismos reguladores de la cáscara, CSE y SCGs como “Nuevos Alimentos”. El uso de subproductos del café como nuevos ingredientes contribuye a la sostenibilidad del sector cafetero, a la reducción de su impacto negativo en el medioambiente, a la mejor utilización de sus recursos naturales y a lograr una nutrición y salud sostenibles para la población mundial.

Annals of Nutrition & Food Science

Review Article
Published: 29 Sep, 2017

Coffee Silverskin: A Low-Cost Substrate for Bioproduction of High-Value Health Promoting Products

*Amaia Iriondo-DeHond[1], Beatriz Fernandez-Gomez[1], Nuria Martinez-Saez[1], Danik M Martirosyan[2], María Dolores Mesa Garcia[3]** and María Dolores del Castillo[1]**

[1]Department of Bioactivity and Food Analysis, Universidad Autónoma de Madrid, Spain

[2]Functional Food Center/Functional Food Institute, USA

[3]Deparment of Biochemistry and Molecular Biology II, University of Granada, Spain

Abstract

In the last decade, the valorization of food wastes has become a priority research line in order to achieve a sustainable food industry. Large amounts of by-products are generated during the coffee industrial processing. Coffee Silverskin (CS) is a thin tegument of the outer layer of the coffee bean and it is the only by-product of the roasting process. This agricultural by-product causes an environmental impact in countries dedicated to its cultivation and processing. Our research group patented an aqueous extract of coffee silverskin (CSE) (P201131128) that is rich in different phytochemicals possessing multifunctional properties such as antioxidant capacity and potential for several applications in nutrition, health and cosmetic. Another priority of our society is to find natural and sustainable strategies to reduce the risk of chronic diseases, in particular those considered epidemics of the 21st century: obesity and diabetes. The aim of the present review is to provide scientific evidence of the usefulness of CSE as a sustainable natural bioproduct for chronic diseases.

Keywords: Antioxidants; Chronic diseases; Coffee by-products; Coffee silverskin; Obesity; Oxidative stress; sustainability

OPEN ACCESS

Correspondence:

*Dolores del Castillo, Department of Bioactivity and Food Analysis, Institute of Food Science Research (CIAL, UAM-CSIC), Nicolás Cabrera, 9 Campus Cantoblanco, Universidad Autónoma de Madrid, 28049 Madrid, Spain, Tel: +34 91 0017900 Ext. 953; E-mail: mdolores.delcastillo@csic.es

**María Dolores Mesa Garcia, Department of Biochemistry and Molecular Biology II, Institute of Nutrition and Food Technology, Centre of Biomedical Research, Laboratory 123, University of Granada, Avenida del Conocimiento s/n, 18100 Armilla, Granada, Spain, Tel: +34 958242334; E-mail: mdmesa@ugr.es

Received Date: 09 Jun 2017
Accepted Date: 22 Sep 2017
Published Date: 29 Jul 2017

Citation:

Iriondo-DeHond A, Fernandez-Gomez B, Martinez-Saez N, Martirosyan DM, Garcia MDM, del Castillo MD. Coffee Silverskin: A Low-Cost Substrate for Bioproduction of High-Value Health Promoting Products. Ann Nutr Food Sci. 2017; 1(1): 1005.

Introduction

Today, 415 million people have diabetes and this alarming number is expected to reach 642 million by 2040. Type 2 Diabetes (T2D) is the most common type of diabetes representing 90% to 95% of all cases. This disease is growing rapidly worldwide in both developed and developing nations. This rise is associated with economic development, ageing populations, increasing urbanization, dietary changes, reduced physical activity and changes in other lifestyle patterns [1].

The term T2D designates not a single disease but a heterogeneous collection of hyperglycemic syndromes resulting from the interaction between a genetic predisposition and behavioral and environmental risk factors. There is strong evidence that obesity and physical inactivity are the main non-genetic determinants of the disease. Usually, T2D occurs in adults, but it is increasingly seen in children and adolescents. The development of T2D is usually associated with a combination of insulin resistance and beta cell failure leading to high blood glucose levels. Insulin resistance is defined as a pathophysiological condition in which a normal insulin concentration does not adequately produce a normal insulin response in peripheral tissues, such as adipose, muscle and liver tissues [2]. Under these conditions, pancreatic beta cells secrete more insulin (i.e. hyperinsulinemia) to overcome the hyperglycemia among insulin-resistant individuals. Although hyperinsulinemia may compensate maintaining normoglycemia, it may cause the over-expression of other insulin activities [3,4].

Nowadays, experimental and clinical studies support the role of oxidative stress in the pathogenesis of T2D [5]. In diabetes, free radical formation by non-enzymatic glycation of proteins, glucose oxidation and increased lipid peroxidation, leads to the damage of enzymes and cellular machinery and also increased insulin resistance [6]. Oxidative stress and free radicals play a major role in the onset and progression of late diabetic complications such as coronary artery disease, neuropathy, nephropathy and retinopathy [7]. *In vivo* studies support the role of hyperglycemia in the enhancement of oxidative stress leading to endothelial dysfunction in blood vessels of diabetic patients [8].

Food has a vital role in maintaining our health properly and in helping in the prevention and

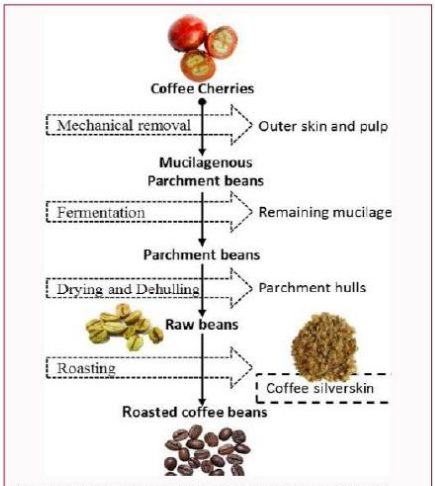


Figure 1: Diagram of coffee silverskin production from wet processing.

cure of some diseases. Nowadays, more than 95% of all chronic disease is caused by food choice, toxic food ingredients, nutritional deficiencies and lack of physical exercise. Many plant extracts and natural compounds are emerging as functional candidates for the reduction of risk of non-communicable chronic diseases, such as T2D [9].

Coffee is considered as an antioxidant beverage with potential beneficial effects on human health [10]. This antioxidant property is due to the presence of bioactive compounds such as caffeine, hydroxycinnamic acids including Chlorogenic Acid (CGA), and melanoidins [11]. Several epidemiological studies have documented the protective effect of coffee components against the risk of chronic diseases due to oxidative stress and inflammation including diabetes [12].

Coffee consumption has been associated with a lower risk of T2D, which may influence different mechanisms such as glucose tolerance, insulin sensitivity, insulin resistance, glucose-6-phosphatase, intestinal glucose absorption, antioxidant activity, inflammatory biomarkers, glucose uptake, glucose homeostasis, glucose metabolism and insulin secretion [13,14]. Although these physiological effects of coffee are related to different components present in the beverage and to the cumulative effects of each compound, most studies on coffee and diabetes clearly associate the observed biological effects to caffeine and CGA [10,13,14].

Coffee silverskin is the thin tegument of the outer layer of the coffee beans and represents about 4.2% (w/w). It is the only by-product produced during the roasting process (Figure 1). This coffee by-product presents phenolic compounds, mainly CGA, and other phytochemicals and bioactive compounds that contribute to its high antioxidant capacity. Our research group patented a CSE from Arabica (*Coffea arabica*) and Robusta (*Coffea canephora*) coffee silverskin (WO 2013004873 A1) enriched in caffeine and CGA [15]. This CSE is extracted with 2 volumes of water per gram of CS at 100ºC for at least 10 min, and does not use organic solvents. Thus, CSE is obtained using an environment-friendly technology [15]. The extraction of bioactive compounds from natural products like CS is increasingly being used to prepare dietary supplements (nutraceuticals), food ingredients and some pharmaceutical products (Figure 2) [16].

The patented CSE is rich in total dietary fiber (28% to 36%), which includes about 4% to 9% insoluble dietary fiber and 24% to 26% soluble dietary fiber. CSEs are a good source of polyphenols, particularly CGA (1% to 6%); the most relevant are 5-O-, 3-O- and 4-O-caffeoylquinic acids [9]. CSE is also a good source of caffeine (3%), and melanoidins (17% to 23%) which are formed during the roasting process [17]. The chemical composition of the patented CSE has been described by [9]. Recent studies [18] have shown that CSE contained 11.42 µg/L of acrylamide; which is approximately 10 times lower than that reported in coffee beverages. After *in vitro* digestion under mimicked human conditions acrylamide was not bioaccesible. Therefore, CS may be used as a safe and natural source of health promoting compounds for chronic diseases.

Coffee Silverskin Extract, Oxidative Stress and Aging

Generally, cells are able to balance the production of oxidants and antioxidants. However, when cells are subjected to excessive levels of ROS or as a result of antioxidant depletion, oxidative stress occurs [19]. Under normal conditions, ROS are natural byproducts produced in mitochondria, peroxisome and in the plasma membrane which have positive physiological effects on cells, such as killing microorganisms, acting as a second messenger (H_2O_2) in cellular differentiation and proliferation and regulating signal transduction [20]. However, ROS are also induced by exogenous sources (UV radiation or chemical agents) and cause DNA, protein and lipid damage. The combination of DNA mutations, protein oxidation and lipid peroxidation induces a cellular progressive decline as a result of insufficient supply of energy leading to oxidative stress-induced aging [21]. In the process of aging, there is a deficiency of the endogenous antioxidant defenses of cells and the residual ROS generate oxidative stress, even in physiological conditions [22]. Large epidemiological studies support the relationship between oxidative state and global health, while high consumption of foods rich in antioxidants is associated with lower disease rates and preventive protection [23]. The anti-aging effect of CSE has been investigated *in vivo* employing as an animal model *Caenorhabditis elegans* [24].

Chlorogenic acid has been described as an anti-aging compound in *C. elegans*. A recent study has demonstrated that CGAs, caffeine, melanoidins and other bioactive compounds all together in the CSE may act in a synergic manner when protecting from UV-induced accelerated aging on *C. elegans* [24]. The nematodes that were treated with CSE (1 mg/mL) showed a significantly increased longevity compared to those cultured on a standard diet. The increased longevity observed was similar to that of the nematodes fed on CGA or vitamin C (0.1 µg/mL). The antiaging properties of the CSE observed in this study are due to its antioxidant character caused by phenols among other bioactive compounds present in the botanical material. Some plant extracts containing CGA and other polyphenols are able to exert an antiaging effect on *C. elegans*. For instance, crude blueberry extract and blueberry polyphenols (including an hydroxycinnamic ester fraction containing CGA) have lengthened the nematode's mean lifespan by 28% [25]. Moreover, Vayndorf et

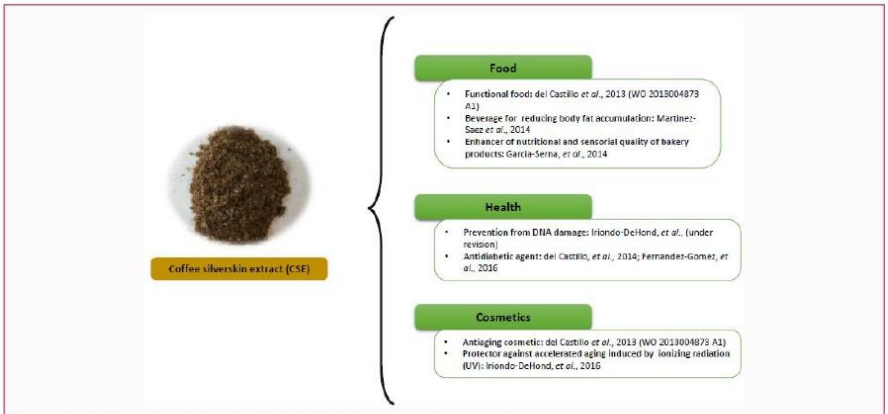


Figure 2: Applications of the patented CSE (WO 2013004873 A1) in food, health and cosmetics.

al. [26] observed that when *C. elegans* was pre-treated with whole apple extracts, worms were more resistant to stresses such as heat, UV radiation and pathogenic infection, suggesting that cellular defense and immune system functions were improved. The authors suggest a possible antioxidant mechanism underlying the antiaging effects of whole apple phytochemicals [26]. Coffee silverskin extract has the potential to be used as an ingredient in skin care products for topical use and as nutricosmetic to prevent accelerated skin aging induced by oxidative stress caused either by exogenous sources (photoaging).

Oxidative stress can also lead to DNA lesions such as DNA strand breaks and oxidized bases [27]. Considering the high antioxidant power of CSE, this extract could protect cells from DNA damage when induced by an oxidative agent. Benzo(a)pyrene (B(a)P) is a carcinogenic Polycyclic Aromatic Hydrocarbon (PAH) found in air, water, soils and in thermally processed foods and cigarette smoke [28,29]. Benzo(a)pyrene induces the production of ROS in cells during the metabolism of this food mutagen, which leads to DNA damage [30].

Have evaluated the protective effect of CSE and CGA against B(a)P induced DNA damage (strand breaks and oxidized purines/pyrimidines) in HepG2 cells. Results showed a significant decrease ($p \leq 0.05$) in DNA strand breaks when cells were pretreated with CSE and CGA [31]. Several authors have confirmed the protective effect of roasted coffee consumption on DNA integrity in humans [32,33]. The reduction of spontaneous DNA strand breaks observed may be attributed to the presence of antioxidants with chemo preventive properties (such as CGAs and roast-associated constituents) [32]. Considering that CS keeps part of the polyphenolic compounds that are normal constituents of coffee beans, such as CGA, it is likely that this effect described for coffee brews is also maintained in CS. These results indicate that CSE protects human cells from DNA strand breaks and oxidative DNA damage effects of B(a)P, and that free CGA or linked to other chemical structures seem to be contributors to the observed chemo protective effect of CSE [31]. This extract presents potential as a natural and sustainable food ingredient.

Coffee Silverskin, Obesity and Dyslipidemia

Overweight and obesity are the major cause of the metabolic syndrome, which is increasing rapidly in modern societies [34]. Therefore, treatment should focus on weight loss by increasing exercise and improving dietary habits; and medical treatment can be used if lifestyle changes are insufficient. Novel foods from natural sources have attracted much attention as potential therapeutic agents in the prevention and treatment of obesity [35].

Recently, the impact of CSE on obesity and diabetes has been evaluated. CSEs from Arabica and Robusta coffees have been used for the preparation of antioxidant novel beverages to study the inhibitory effect on fat accumulation *in vivo* using as animal model *C. elegans* [36]. A significant dose-response effect on reducing accumulation of body fat was found for pure CGA (3.54 mg/L) and caffeine (4.85 mg/L), achieving 30% and 29% reduction of lipid deposits, respectively. The brews of Arabica and Robusta CSE (100 µg/mL), which contained physiologically active doses of CGA and caffeine, were effective reducing body fat 21% and 24%, respectively. Furthermore, similar results in body fat reduction by Robusta CSE beverage were found when a commercial dietary supplement made from Robusta decaffeinated green coffee extract was studied. Therefore, CSE is a natural alternative to dietary supplements for the prevention of overweight and obesity [18,36].

In addition, CSE reduced total cholesterol and triglycerides plasma levels in rats after 45 days of treatment with CSE (2.2 mg caffeine/kg body weight and 0.8 mgCGA/kg body weight). CSE also reduced 41.73% the activity of pancreatic lipase *in vitro* at concentration of 36 mg/mL. This could explain the mechanism of action of the observed reduction of total cholesterol and triglycerides, since pancreatic lipase is a key enzyme in fat digestion [37]. These results support the lyporegulatory character of CSE through the inhibition of pancreatic lipase and therefore its preventive and therapeutic effect in the obesity disease.

The anti-obesity effect of coffee may be due to its bioactive compounds, such as caffeine, CGAs and melanoidins, which are

also present in coffee silverskin [17,37]. CGA and caffeine can regulate lipid metabolism by modulating cell signaling, reducing lipid accumulation and size of adipocytes [38], inhibiting pancreatic lipase [39], regulating hepatic lipid metabolism-related enzymes [40], and by down regulating genes involved in adipogenesis [41]. The combination of these effects leads to the suppression of body fat accumulation [42]. In addition, coffee melanoidins have showed to protect against non-alcoholic fatty liver disease by reducing the hepatic fat accumulation in the rat model [43].

Coffee Silverskin and Diabetes

Type 2 diabetes is usually associated to a combination of insulin resistance and beta cell failure leading to high blood glucose levels. Hyperglycemia is a major factor contributing to accelerated protein glycation and the formation of Advanced Glycation End Product (AGEs) [44]. In diabetes, free radical formation by non-enzymatic glycation of proteins, glucose oxidation and increased lipid peroxidation, leads to damage of enzymes, cellular machinery and also increases insulin resistance [6]. Oxidative stress plays a major role in the development of late diabetic complications such as coronary artery disease, neuropathy, nephropathy, and retinopathy [7].

Studies suggest that moderate intake of coffee may lower risk of T2D [45]. The effects observed on diabetes biomarkers may be associated to the synergic effect of different bioactive compounds present in coffee such as CGA, caffeine, their metabolites and others coffee components. Some of these compounds are also present in CSE [9] and have an effect in diabetes biomarkers.

Caffeine concentrations present in CSE range between 3% and 3.4% [9]. Studies performed in rats suffering streptozotocin-induced diabetes showed that caffeine in CSE was metabolized and the metabolites protected the pancreas against oxidative stress [46]. In addition, caffeine can also reduce glucose levels and insulin sensitivity [47,48]. Other authors have also observed a protective effect of caffeine in pancreatic beta cells [49,50].

Coffee silverskin extract also contains CGA in a range of 1.1% to 6.8% [9]. Results obtained by our research group suggest that CGA and its metabolites have a greater effect on T2D biomarkers than caffeine. CGA and its roasting-formed derivates present in CSE have been proposed as the main contributors to the beneficial effects of CSE on T2D [14,51]. The different mechanisms by which CGA exerts its antidiabetic effect are: a) regulation of glucose metabolism [45,52], b) enhancement of insulin action [52,53]. c) Inhibition of α-glucosidase activity [54,55], d) protection against oxidative stress [56] and e) inhibition of AGEs formation [57]. Different studies demonstrate that the formation of fluorescent AGEs is inhibited by different pathways such as carbonyl trapping, antioxidant effect and the formation of protein-phenols conjugates [57-59].

Other coffee constituents relevant in the prevention of T2D are melanoidins, melatonin, lignans and lignin, tannic acid, isoflavones and trigonelline, all of which may be present in CSE. These compounds may exert synergic effects being responsible for the health-promoting properties of CSE. Further research should be carried out in order to confirm the presence of these compounds in CSE and to prove their effect in the prevention of T2D.

In conclusion, CSE contains a number of coffee components able to reduce the risk of accelerated aging and chronic metabolic disorders such as T2D. These effects may be associated to its antioxidant power and capacity to inhibit enzymes involved in the metabolism of nutrients.

Acknowledgements

This research was supported by the NATURAGE (AGL2010-17779) and SUSCOFFEE (AGL2014-57239-R) projects. Fernandez-Gomez, B. (BES-2011-046827) and Iriondo-DeHond, A. (BES-2015-072191), thanks the Spanish Ministry of Economy and Competitiveness (MINECO) for their pre-doctoral research fellowship (FPI) and Martinez-Saez, N. thanks the Autonomous University of Madrid (U.A.M) for her FPI-predoc fellow.

References

1. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. Nature. 2001;414(6865):782-7.
2. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev. 2013;93(1):137-88.
3. American Diabetes Association. Classification and diagnosis of diabetes. Diabetes Care. 2016;39:S13-22.
4. Hameed I, Masoodi SR, Mir SA, Nabi M, Ghazanfar K, Ganai BA. Type 2 diabetes mellitus: From a metabolic disorder to an inflammatory condition. World J Diabetes. 2015;6(4):598-612.
5. Nathan DM, Davidson MB, DeFronzo RA, Heine RJ, Henry RR, Pratley R, et al. Impaired fasting glucose and impaired glucose tolerance. Diabetes Care. 2007;30(3):753-9.
6. Maritim AC, Sanders RA, Watkins JB. Diabetes, oxidative stress, and antioxidants: A review. J Biochem Mol Toxicol. 2003;17(1):24-38.
7. Oguntibeju O. Antioxidant-antidiabetic agents and human health. Croatia: InTech; 2014.
8. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010;107(9):1058-70.
9. del Castillo MD, Fernandez-Gomez B, Martinez-Saez N, Iriondo-DeHond A, Martirosyan DM, Mesa MD. Coffee silverskin extract for aging and chronic diseases. In: Martirosyan DM, editor. Functional Foods in Health and Disease. CreateSpace Independent Publishing Platform; 2016.
10. Moreira ASP, Nunes FM, Domingues MR, Coimbra MA. Coffee melanoidins: structures, mechanisms of formation and potential health impacts. Food Funct. 2012;3:903-15.
11. Fava F, Totaro G, Diels L, Reis M, Duarte J, Carioca OB, et al. Bio waste bio refinery in Europe: opportunities and research & development needs. N Biotechnol. 2015;32(1):100-8.
12. Bisht S, Sisodia S. Coffea arabica: A wonder gift to medical science. J Nat Pharm. 2010;1(1):58-65.
13. Akash MS, Rehman K, Chen S. Effects of coffee on type 2 diabetes mellitus. Nutrition. 2014;30(7-8):755-63.
14. Ludwig IA, Clifford MN, Lean MEJ, Ashihara H, Crozier A. Coffee: biochemistry and potential impact on health. Food Funct. 2014;5(8):1695-717.
15. del Castillo MD, Ibañez ME, Amigo M, Herrero M, Plaza del Moral M, Ullate M. Application of products of coffee silverskin in anti-ageing cosmetics and functional food. 2013.
16. Dai J, Mumper RJ. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules. 2010;15(10):7313-52.
17. Mesías M, Navarro M, Martínez-Saez N, Ullate M, del Castillo MD, Morales FJ. Antiglycative and carbonyl trapping properties of the water soluble fraction of coffee silverskin. Food Res Int. 2014;62:1120-6.
18. Garcia-Serna E, Martinez-Saez N, Mesias M, Morales FJ, del Castillo MD.

Use of coffee silverskin and stevia to improve the formulation of biscuits. Polish J Food Nutr Sci. 2014;64(4):243-51.

19. Hancock JT, Desikan R, Neill SJ. Role of reactive oxygen species in cell signalling pathways. Biochem Soc Trans. 2001;29:345-50.
20. Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol. 2010;38(1):96-109.
21. Baumann L. Skin ageing and its treatment. J Pathol. 2007;211(2):241-51.
22. Momtaz S, Abdollahi M. A comprehensive review of biochemical and molecular evidences from animal and human studies on the role of oxidative stress in aging: An epiphenomenon or the cause. Asian J Anim Vet Adv. 2012;7(1):1-19.
23. Prior RL. Oxygen radical absorbance capacity (ORAC): New horizons in relating dietary antioxidants/bioactives and health benefits. J Funct Foods. 2015;18:797-810.
24. Iriondo-DeHond A, Martorell P, Genovés S, Ramón D, Stamatakis K, Fresno M, et al. Coffee silverskin extract protects against accelerated aging caused by oxidative agents. Molecules. 2016;21(6):1-14.
25. Wilson MA, Shukitt-Hale B, Kalt W, Ingram DK, Joseph JA, Wolkow CA. Blueberry polyphenols increase lifespan and thermo tolerance in Caenorhabditis elegans. Aging Cell. 2006;5(1):59-68.
26. Vayndorf EM, Lee SS, Liu RH. Whole apple extracts increase lifespan, health span and resistance to stress in Caenorhabditis elegans. J Funct Foods. 2013;5(3):1236-43.
27. Xue W, Warshawsky D. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. Toxicol Appl Pharmacol. 2005;206(1):73-93.
28. Brinkmann J, Stolpmann K, Trappe S, Otter T, Genkinger D, Bock U. Metabolically competent human skin models: activation and genotoxicity of benzo[a]pyrene. Toxicol Sci. 2013;131(2):351-9.
29. International Agency for Research on Cancer. Chemical agents and related occupations. In: A review of human carcinogens. Lyon: IARC, France; 2012.
30. Haza AI, Morales P. Spanish honeys protect against food mutagen-induced DNA damage. J Sci Food Agric. 2013;93(12):2995-3000.
31. Iriondo-DeHond A, Haza AI, Avalos A, del Castillo MD, Morales P. Validation of coffee silverskin extract as a food ingredient by the analysis of cytotoxicity and genotoxicity. Food Res Int. 2017;100:791-7.
32. Bakuradze T, Lang R, Hofmann T, Eisenbrand G, Schipp D, Galan J, et al. Consumption of a dark roast coffee decreases the level of spontaneous DNA strand breaks: a randomized controlled trial. Eur J Nutr. 2014;54(1):149-56.
33. Bakuradze T, Boehm N, Janzowski C, Lang R, Hofmann T, Stockis JP, et al. Antioxidant-rich coffee reduces DNA damage, elevates glutathione status and contributes to weight control: Results from an intervention study. Mol Nutr Food Res. 2011;55(5):793-7.
34. Tsai YJ, Wu MP, Hsu YW. Emerging health problems among women: Inactivity, obesity, and metabolic syndrome. Gynecol Minim Invasive Ther. 2014;3(1):12-14.
35. Boqué N, Campión J, de la Iglesia R, de la Garza AL, Milagro FI, San Román B, et al. Screening of polyphenolic plant extracts for anti-obesity properties in Wistar rats. J Sci Food Agric. 2013;93(5):1226-32.
36. Martinez-Saez N, Ullate M, Martin-Cabrejas MA, Martorell P, Genovés S, Ramon D. A novel antioxidant beverage for body weight control based on coffee silverskin. Food Chem. 2014;150:227-34.
37. del Castillo MD, Fernandez-Gomez B, Ullate M, Mesa MD. Use of coffee husk products for the prevention and treatment of the pathologies that make up the metabolic syndrome and its metabolic factors. Risk. 2014;P201431848.
38. Kim J, Jang JY, Cai J, Kim Y, Shin K, Choi EK, et al. Ethanol extracts of unroasted Coffea canephora robusta beans suppress adipogenesis in preadipocytes and fat accumulation in rats fed a high-fat diet. Food Sci Biotechnol. 2014;23(6):2029-35.
39. Narita Y, Iwai K, Fukunaga T, Nakagiri O. Inhibitory activity of chlorogenic acids in decaffeinated green coffee beans against porcine pancreas lipase and effect of a decaffeinated green coffee bean extract on an emulsion of olive oil. Biosci Biotechnol Biochem. 2012;76(12):2329-31.
40. Zheng G, Qiu Y, Zhang QF, Li D. Chlorogenic acid and caffeine in combination inhibit fat accumulation by regulating hepatic lipid metabolism-related enzymes in mice. Br J Nutr. 2014;112(6):1034-40.
41. Song SJ, Choi S, Park T. Decaffeinated green coffee bean extract attenuates diet-induced obesity and insulin resistance in mice. Evid Based Complement Altern Med. 2014;2014:718379.
42. Murase T, Misawa K, Minegishi Y, Aoki M, Ominami H, Suzuki Y, et al. Coffee polyphenols suppress diet-induced body fat accumulation by down regulating SREBP-1c and related molecules in C57BL/6J mice. Am J Physiol Endocrinol Metab. 2011;300(1):E122-33.
43. Vitaglione P, Morisco F, Mazzone G, Amoruso DC, Ribecco MT, Romano A, et al. Coffee reduces liver damage in a rat model of steatohepatitis: the underlying mechanisms and the role of polyphenols and melanoidins. Hepatology. 2010;52(5):1652-61.
44. Vlassara H, Uribarri J. Advanced glycation end products (AGE) and diabetes: cause, effect, or both? Curr Diab Rep. 2014;14(1):453.
45. van Dijk AE, Olthof MR, Meeuse JC, Seebus E, Heine RJ, van Dam RM. Acute effects of decaffeinated coffee and the major coffee components chlorogenic acid and trigonelline on glucose tolerance. Diabetes Care. 2009;32(6):1023-5.
46. Fernandez-Gomez B, Lezama A, Amigo-Benavent M, Ullate M, Herrero M, Martin MA, et al. Insights on the health benefits of the bioactive compounds of coffee silverskin extract. J Funct Foods. 2016;25:197-207.
47. Kagami K, Morita H, Onda K, Hirano T, Oka K. Protective effect of caffeine on streptozotocin-induced beta-cell damage in rats. J Pharm Pharmacol. 2008;60(9):1161-5.
48. Urzua Z, Trujillo X, Huerta M, Trujillo-Hernandez B, Rios-Silva M, Onetti C, et al. Effects of chronic caffeine administration on blood glucose levels and on glucose tolerance in healthy and diabetic rats. J Int Med Res. 2012;40(6):2220-30.
49. Abunasef SK, Amin HA, Abdel-hamid GA. A histological and immunohistochemical study of beta cells in streptozotocin diabetic rats treated with caffeine. Folia Histochem Cytobiol. 2014;52(1):42-50.
50. Chen L, Yu M, Shen T, Xia J, Xu BL. Impact of caffeine on β cell proliferation and apoptosis under the influence of palmitic acid. Genet Mol Res. 2015;14(2):5724-30.
51. Cano-Marquina A, Tarín JJ, Cano A. The impact of coffee on health. Maturitas. 2013;75(1):7-21.
52. Li SY, Chang CQ, Ma FY, Yu CL. Modulating effects of chlorogenic acid on lipids and glucose metabolism and expression of hepatic peroxisome proliferator-activated receptor-alpha in golden hamsters fed on high fat diet. Biomed Environ Sci. 2009;22(2):122-9.
53. Ma Y, Gao M, Liu D. Chlorogenic acid improves high fat diet-induced hepatic steatosis and insulin resistance in mice. Pharm Res. 2015;32(4):1200-9.
54. Ishikawa A, Yamashita H, Hiemori M, Inagaki E, Kimoto M, Okamoto M, et al. Characterization of inhibitors of postprandial hyperglycemia from the leaves of Nerium indicum. J Nutr Sci Vitaminol (Tokyo). 2007;53(2):166-73.
55. Tateishi E, Han LK, Okuda H. Effect of a hot-water extract from coffee beans on the postprandial blood glucose concentration in rats. Japanese J Nutr Diet. 2004;62:323-7.

Annex 1

56. Karthikesan K, Pari L, Menon VP. Combined treatment of tetrahydrocurcumin and chlorogenic acid exerts potential antihyperglycemic effect on streptozotocin-nicotinamide-induced diabetic rats. Gen Physiol Biophys. 2010;29(1):23-30.

57. Kim J, Jeong IH, Kim CS, Lee YM, Kim JM, Kim JS. Chlorogenic acid inhibits the formation of advanced glycation end products and associated protein cross-linking. Arch Pharm Res. 2011;34(3):495-500.

58. Fernandez-Gomez B, Ullate M, Picariello G, Ferranti P, Mesa MD, del Castillo MD. New knowledge on the antiglycoxidative mechanism of chlorogenic acid. Food Funct. 2015;6(6):2081-90.

59. Gugliucci A, Bastos DH, Schulze J, Souza MF. Caffeic and chlorogenic acids in Ilex paraguariensis extracts are the main inhibitors of AGE generation by methylglyoxal in model proteins. Fitoterapia. 2009;80(6):339-44.

Bionok®

Regulatory services & quality systems

Drugs, Medical Devices, Food & Supplements, Cosmetics, Substances, Biocides, Detergency, Biosimilars

Certifica

que

Alodia Farmaceútica, S.l.

Ha encargado a Bionok Healthcare, S.l. el registro como "Novel Food" ante la EFSA de acuerdo al reglamento 2283/2015/UE relativo a los nuevos alimentos, de la invención "Silverskin" cuya nomenclatura inci es: "Coffea Arabica/Robusta chaff Extract". Patente desarrollada por el equipo de la Dr. María Dolores del Castillo Bilbao del Instituto de Investigación en Ciencias de la Alimentación (CIAL), y sobre el cual está realizando el doctorado Dña. Amaia Iriondo de Hond, y cuyo tramite de registro una vez elaborada la documentación pertinente será realizado en el mes de octubre del presente.

martes, 1 de octubre de 2019

CEO Bionok Heathcare, SL.

Bionok Healthcare, S.l. NIF B87444733. Representative Office: Calle Ribera del Loira, 46 28042 Madrid
Technical Office. C/Doménico Veneciano, 5 – local 1 Telf: 0034 91 137 11 70 www.bionok.eu – info@bionok.eu

molecules

MDPI

Article

Coffee Silverskin Extract Protects against Accelerated Aging Caused by Oxidative Agents

Amaia Iriondo-DeHond [1], Patricia Martorell [2], Salvador Genovés [2], Daniel Ramón [2], Konstantinos Stamatakis [3], Manuel Fresno [3], Antonio Molina [4] and Maria Dolores del Castillo [1,*]

1 Institute of Food Science Research (CSIC-UAM), Nicolás Cabrera 9, 28049 Madrid, Spain; amaia.iriondo@csic.es

2 Biópolis SL, Parc Científic Universitat de Valencia, Catedrático Agustín Escardino 9, edificio 2, 46980 Valencia, Spain; patricia.martorell@biopolis.es (P.M.); salvador.genoves@biopolis.es (S.G.); daniel.ramon@biopolis.es (D.R.)

3 Centro de Biología Molecular Severo Ochoa (CBM-SO) (CSIC-UAM), Nicolás Cabrera, 1, 28049 Madrid, Spain; kstamatakis@cbm.csic.es (K.S.); mfresno@cbm.csic.es (M.F.)

4 Beacon Biomedicine, Parque Científico de Madrid, Santiago Grisolia 2, D130/L145, 28760 Madrid, Spain; amolina@advmedprojects.com

* Correspondence: mdolores.delcastillo@csic.es; Tel.: +34-91-0017-900

Academic Editor: Pedro Mena
Received: 14 April 2016; Accepted: 20 May 2016; Published: 1 June 2016

Abstract: Nowadays, coffee beans are almost exclusively used for the preparation of the beverage. The sustainability of coffee production can be achieved introducing new applications for the valorization of coffee by-products. Coffee silverskin is the by-product generated during roasting, and because of its powerful antioxidant capacity, coffee silverskin aqueous extract (CSE) may be used for other applications, such as antiaging cosmetics and dermaceutics. This study aims to contribute to the coffee sector's sustainability through the application of CSE to preserve skin health. Preclinical data regarding the antiaging properties of CSE employing human keratinocytes and *Caenorhabditis elegans* are collected during the present study. Accelerated aging was induced by *tert*-butyl hydroperoxide (*t*-BOOH) in HaCaT cells and by ultraviolet radiation C (UVC) in *C. elegans*. Results suggest that the tested concentrations of coffee extracts were not cytotoxic, and CSE 1 mg/mL gave resistance to skin cells when oxidative damage was induced by *t*-BOOH. On the other hand, nematodes treated with CSE (1 mg/mL) showed a significant increased longevity compared to those cultured on a standard diet. In conclusion, our results support the antiaging properties of the CSE and its great potential for improving skin health due to its antioxidant character associated with phenols among other bioactive compounds present in the botanical material.

Keywords: coffee silverskin; oxidative stress; UVC radiation; chlorogenic acid; skin health; accelerated aging; nutricosmetic; dermaceutic

1. Introduction

Oxidative stress is a major cause of skin accelerated aging and diseases, which is defined as the imbalance between reactive oxygen species (ROS) and antioxidants. Generally, cells are able to balance the production of oxidants and antioxidants. However, when cells are subjected to excessive levels of ROS or as a result of antioxidant depletion, oxidative stress occurs [1]. Under normal conditions, ROS are natural byproducts produced in mitochondria, the peroxisome and the plasma membrane, which have positive physiological effects on cells, such as killing microorganisms, acting as a second messenger in cellular differentiation and proliferation and regulating signal transduction [2]. However, ROS can also be generated by exogenous sources (UV radiation or chemical agents) and cause DNA, protein and lipid damage. This can lead to skin diseases, such as dermatitis, sunburn, acne, eczema,

vasculitis, psoriasis and cancer [3]. Damage caused by oxidative stress can be studied in *ex vivo* models [4] or *in vivo* models [5] using the biomarkers described in Figure 1.

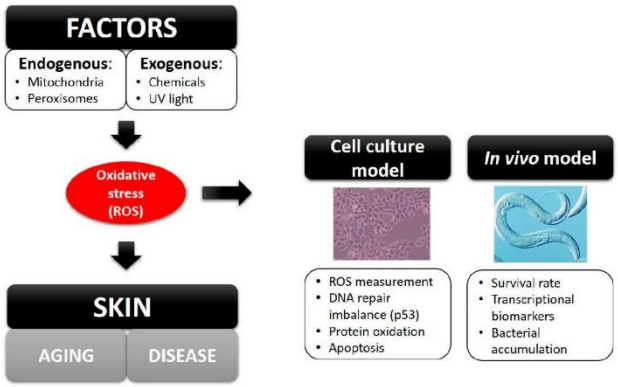


Figure 1. Biomarkers used in cell culture models or *in vivo* models to study the effects of oxidative stress.

Coffee beverage is known for the antioxidant properties of its components, such as caffeine, chlorogenic acid (CGA), hydroxycinnamic acids and melanoidins [6]. In the preparation of this beverage, over 90% of the raw material is discarded as an agricultural by-product. The valorization of such wastes using the biorefinery approach represents a real contribution of many industries for sustainable and competitive development [7].

Many plant extracts and natural compounds are emerging as candidates for the protection of the effects of UV-induced damage on skin; for instance, resveratrol [8,9], citrus and rosemary extract [10] or *Castanea sativa* extract [11]. Various studies suggest that coffee extracts can protect skin cells against photoaging induced by UV irradiation, as well [12–14]. In this context, the biomass resulting from coffee roasting (coffee silverskin) could go through biorefinery processes for use as a bioactive compound as a "dermaceutical" in cosmetics. The interest of using coffee silverskin aqueous extract (CSE) in cosmetics was proposed for the first time by del Castillo *et al.* in a patent application filed in 2011, which became public in 2013 (WO/2013/004873) [15]. Very recently, Rodrigues *et al.* tested the *in vitro* antioxidant and antimicrobial capacity of CSE and its cytotoxicity in human skin cells [16]. However, CSE's ability to protect from sun radiation has not been studied yet. Health benefits of CSE have been associated with its complex and particular chemical composition in bioactive compounds, such as chlorogenic acid (CGA), caffeine, melanoidins and dietary fiber, among others [15,17].

A standard keratinocyte cell culture monolayer can be used to simulate the physiology of the epidermal layer of skin [18]. Such human-derived *in vitro* models are of extreme value for the study of the potential health effects of bioactive compounds on skin when used by topical administration [19]. To the best of our knowledge, the potential of aqueous CSE to reinforce the antioxidant defense of human skin cells has not been previously reported, and it is one of the main goals of the present study. However, since cells grown in monolayers cannot capture the relevant complexity of the *in vivo* microenvironment [19], it is interesting to study the effect of such compounds *in vivo*.

Many biological processes are conserved between humans and *C. elegans*. This nematode has been widely used in aging studies for two reasons: it is a multicellular organism with a fully-sequenced genome, and it has a short lifespan. This nematode is also revealed to have evolutionarily-conserved pathways for aging [20]. In this context, *C. elegans* is the ideal model, since it combines topical and

oral antioxidant administration, which is the favored recommendation [21]. Additionally, *C. elegans* is becoming a fast and inexpensive *in vivo* tool for the cosmetic and pharmaceutical industries for compound screening. There is no ethical problem in the use of *C. elegans*, as this nematode is not regarded as an animal in the EU regulation (Directive 2010/63/EU), and the results obtained are consistent with higher animal models, which enable subsequent pre-clinical and clinical trials to be more oriented. No previous studies on the nutricosmetic antiaging effect of CSE using animal models have been published.

The aim of this study is to evaluate the feasibility of CSE to preserve skin health and to reduce the risk of accelerated aging and skin diseases due to oxidative stress induced by physical and chemical agents. The final intention of the investigation is to contribute to the coffee sector's sustainability through the implementation of the biorefinery concept. Preclinical data regarding the antiaging properties of CSE employing human keratinocytes (HaCaT cells) and *Caenorhabditis elegans* as the animal model are collected during the present study. To do this, accelerated aging was induced by *t*-BOOH (0.5 mM) in HaCaT cells and by UVC in *C. elegans*.

2. Results

2.1. Study of Coffee Silverskin in HaCaT Cells

Prior to the evaluation of the effect of CSE on cells, we first evaluated the *in vitro* antioxidant capacity of the CSE by the $ABTS^{\bullet+}$ radical cation decolorization assay. An overall antioxidant capacity value of 319.3 CGA equivalents (μmol)/gram of CSE and an IC_{50} value of 373.4 μg/mL were obtained for the trapping capacity of cationic free radicals of CSE (Figure S1, Supplementary Materials). These results demonstrate that the patented CSE possesses *in vitro* antioxidant properties.

Then, we determined the cytotoxic effect of CSE (0.01 mg/mL, 0.1 mg/mL 0.5 mg/mL and 1 mg/mL), CGA (6.88 μg/mL), caffeine (19.86 μg/mL) and vitamin C (0.1 μg/mL) on HaCaT cells using the 3-(4,5-dimethylthiazole-y)-2,5-diphenyltetrazolium (MTT) assay. The concentrations of CGA and caffeine used in this study are equivalent to those present in 1 mg/mL of CSE [22]. No significant decrease ($p > 0.05$) of absorbance was observed after incubation of the compounds when cell viability was measured (Figure S2, Supplementary Materials). These results suggest that CSE, CGA, caffeine and vitamin C at the concentrations tested in this investigation have no adverse effects on the viability of HaCaT cells.

In order to study the response of HaCaT cells to oxidative treatment, we initially determined their sensitivity to increasing concentrations of *t*-BOOH by measuring cell viability using the MTT assay (Figure 2). Therefore, cells were treated with different concentrations of *t*-BOOH used previously by Kučera *et al.* (2014) [23] (0.1 mM, 0.25 mM, 0.5 mM and 1 mM), and viability was estimated after 1, 6 and 24 h. The obtained results showed no significant cell viability reduction ($p > 0.05$) when *t*-BOOH was added for one hour. However, cell viability decreased following treatment with *t*-BOOH for 6 and 24 h in a dose-dependent manner. The lowest concentration of *t*-BOOH (0.1 mM) did not reduce cell viability; however, higher concentrations of *t*-BOOH (0.25 mM, 0.5 mM and 1 mM) were cytotoxic to HaCaT cells, since cell viability was significantly reduced ($p < 0.05$) (Figure 2).

Since the *t*-BOOH concentration of 0.5 mM at 6 h caused a significant decrease ($p < 0.05$) in the cell viability of nearly 60% (Figure 2), we decided to choose this concentration to induce oxidative stress in the following experiments.

As we were concerned about the combined effects of *t*-BOOH and CSE on HaCaT cytotoxicity, cells were pre-treated with various doses of CSE for 24 h prior to the induction of oxidative stress with 0.5 mM *t*-BOOH. For cell viability determinations, after pre-treatment with different concentrations of CSE (0.01 mg/mL, 0.1 mg/mL, 0.5 mg/mL and 1 mg/mL), keratinocytes were exposed to 0.5 mM *t*-BOOH for 6 and 24 h. After 6 h of oxidative damage, cell viability decreased significantly ($p < 0.05$) by nearly 60%, in line with the observed results of previous experiments (Figure 3A). When cells were pre-treated with CSE for 24 h prior to oxidation, cell death was diminished when the extract doses

used were 0.5 mg/mL and 1 mg/mL. Since there is no significant difference ($p > 0.05$) between control cells and cells pre-treated with 1 mg/mL of CSE, we can suggest that this dose of CSE fully protected cells from death induced by oxidative stress (Figure 3A).

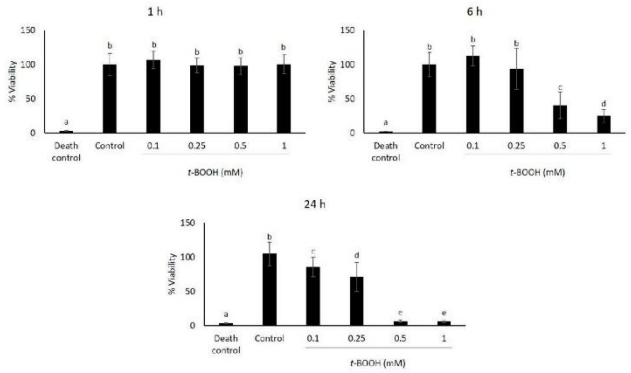


Figure 2. Cell viability determined by the MTT assay of HaCaT cells exposed to different concentrations of *t*-BOOH. Triton X-100 (10%) was used as the death control. Absorbance was measured after 24 h of *t*-BOOH exposure. Data are expressed as the mean of 18 replicates ± SD. Treatments with different letters differ significantly (Tukey test, $p < 0.05$).

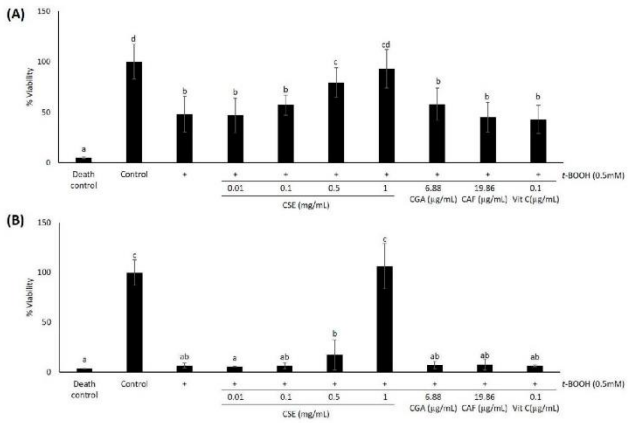


Figure 3. Effect of coffee silverskin extract (CSE), chlorogenic acid (CGA), caffeine (CAF) and vitamin C (Vit C) against oxidative damage induced by *t*-BOOH 0.5 mM. Cells were treated with 0.01–1 mg/mL CSE, 6.88 µg/mL of CGA, 19.86 µg/mL of CAF and 0.1 µg/mL of Vit C for 24 h and further exposed to 0.5 mM *t*-BOOH for 6 h (**A**) or 24 h (**B**). Triton X-100 (10%) was used as the death control. Then, cell viability was measured using the MTT assay. Data represent means ± SD of 18 samples per condition. Different letters denote statistically-significant differences between all treatments ($p < 0.05$).

In order to find out if CSE is able to protect cells when oxidation takes place during 24 h, we performed the MTT assay after 24 h of *t*-BOOH 0.5 mM incubation (Figure 3B). In this case, *t*-BOOH-induced oxidation reduced cell viability in a similar way as the death control ($p > 0.05$). Pre-treatment with 1 mg/mL of CSE was the only dose able to protect cells from such extreme cellular damage. No significant differences ($p > 0.05$) were found between control cells and cells pre-treated with 1 mg/mL of CSE. In none of the cases did CGA, caffeine at concentrations equivalent to those present in 1 mg/mL of CSE and vitamin C have a significant effect in the prevention of *t*-BOOH-induced cell death.

Figure 4 illustrates the effect of the CSE on the appearance of the HaCaT cell monolayer. *t*-BOOH treatment led to morphological changes, such as cell shrinkage related to cell death. However, pre-treatment with CSE 1 mg/mL prevented these morphological alterations.

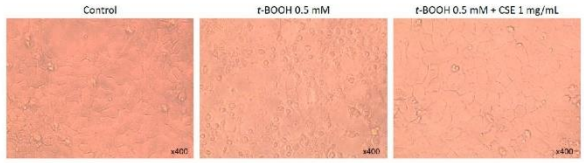


Figure 4. Representative microscopy images (×40) of HaCaT cells after different treatments. Control = untreated cells; *t*-BOOH 0.5 mM = cells treated with 0.5 mM *t*-BOOH for 24 h; *t*-BOOH 0.5 mM; CSE 1 mg/mL = cells pre-treated with 1 mg/mL CSE for 24 h and further exposed to 0.5 mM *t*-BOOH for 24 h.

Considering the prevention of ROS generation, HaCaT cells were incubated with *t*-BOOH 0.5 mM for 1 h, and then intracellular ROS were measured using the 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe (Figure 5). When *t*-BOOH 0.5 mM was added, intracellular ROS significantly increased ($p < 0.05$) from physiological ROS (100%) to 150% approximately. However, when cells were pre-treated with 1 mg/mL of CSE, ROS were diminished to physiological levels ($p > 0.05$). Neither lower concentrations of CSE nor CGA, CAF at concentrations equivalent to those present in 1 mg/mL of CSE and Vit C had an effect in the prevention of oxidative stress, since no significant differences were found between them and non-pre-treated cells ($p > 0.05$). Taking into account the obtained results from the cell culture experiments, HaCaT cells pre-treated with CSE exhibited a marked resistance to *t*-BOOH-induced oxidative damage (Figures 3–5).

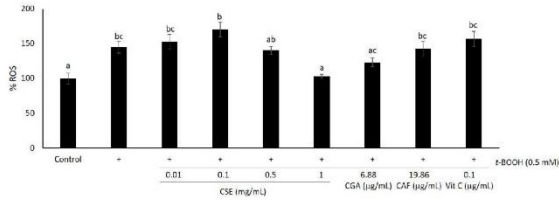


Figure 5. Effect of CSE (0.01–1 mg/mL), CGA (6.88 µg/mL), CAF (19.86 µg/mL) and Vit C (0.1 µg/mL) against oxidative damage induced by *t*-BOOH 0.5 mM. Cells were pre-treated with CSE, CGA, CAF and Vit C for 24 h, incubated with the 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe for 30 min and further exposed to 0.5 mM *t*-BOOH for 1 h. Then, the fluorescence of intracellular ROS was measured. Data represent the means ± SEM of 18 samples per condition. Different letters denote statistically-significant differences between all treatments ($p < 0.05$).

2.2. Study of Coffee Silverskin in *C. elegans*

In order to study the *in vivo* antiaging effect of CSE, *C. elegans* under UVC-induced oxidative stress was fed on different concentrations of CSE (0.01 mg/mL, 0.1 mg/mL and 1 mg/mL), CGA (0.1 µg/mL) and vitamin C (0.1 µg/mL) as controls. *C. elegans* has been widely used in many studies as a model to determine the benefits of different compounds and plant extracts on aging-related parameters. In this case, we used wild-type nematodes (N2 strain) grown in nematode growth (NG) medium with CSE.

With regard to the *in vivo* experiments, CSE was studied in the same concentrations used on HaCaT cells. Nematodes were subjected to a daily UVC treatment (45 s/day) to induce oxidative damage. Thus, UVC treatment provoked a dramatic viability decrease in nematodes as compared to the control conditions (Nematode growth medium without UVC treatment) (Figure 6A,B). This is in accordance with previous reports suggesting an accumulation of DNA damage and a drop of the worm's survival during chronic UV exposition [24]. Moreover, under UVC treatment, it was determined that the dose of 1 mg/mL of CSE showed an increase in nematodes' longevity compared to the control conditions (Figure 6A), and in a similar way as vitamin C and CGA used as positive controls (Figure 6B, Table 1). In fact, this increase proved to be significant with a confidence level of 99% (Table 1). These results suggest the protective effect of CSE on the oxidative stress produced by UVC radiation. In relation to the lower doses of CSE, no effect on longevity was observed (Figure 6A, Table 1).

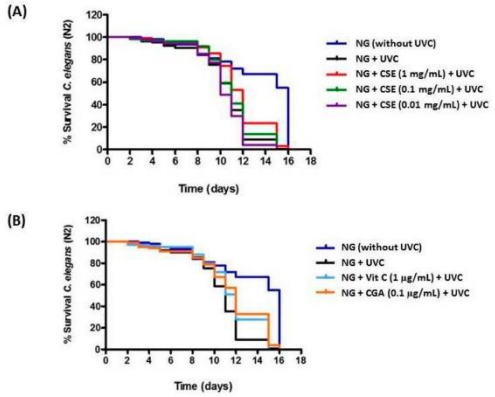


Figure 6. Survival curves of *C. elegans* wild-type strain N2, growing in NG medium supplemented with CGA (0.1 µg/mL) and vitamin C (0.1 µg/mL), as positive controls (**A**) and with CSE (1 mg/mL, 0.1 mg/mL and 0.01 mg/mL) (**B**). Nematodes were treated daily with UVC. A control condition NG without UVC treatment was included. Experiments were performed in triplicate.

Table 1. Effect of CSE on *C. elegans* lifespan with UVC treatment.

Strain	Treatment	Life Expectancy (50%) (Days)	*p*-Value
N2	NG	11	
	NG + Vit C (0.1 µg/mL)	12	0.0021 **
	NG + CGA (0.1 µg/mL)	12	0.0003 ***
	NG + CSE (1 mg/mL)	12	0.0013 **
	NG + CSE (0.1 mg/mL)	11	0.3364 (NS)
	NG + CSE (0.01 mg/mL)	10	0.2133 (NS)

NG = nematode growth; Vit C = vitamin C; CGA = chlorogenic acid; CSE = coffee silverskin extract; NS: not significant; ** = $p < 0.05$; *** = $p < 0.001$.

3. Discussion

The aim of this study was to obtain novel information regarding the use of CSE and its bioactive compounds, CGA and caffeine, on accelerated aging and skin damage induced by oxidative stress. For this purpose, we used an established human cell culture line (HaCaT cells) as a skin model and *C. elegans* as an animal model.

In our study, values of the overall antioxidant capacity of CSE agree with those reported by Mesías *et al.* [25] and Fernandez-Gomez *et al.* [26]. Results demonstrate that our CSE has powerful antioxidant capacity, in a similar way to other CSE reported by Narita and Inouye [27], del Castillo *et al.* [15] and Borrelli *et al.* [28]. CSE antioxidant capacity may be explained by the presence of polyphenolic compounds, such as chlorogenic acid and melanoidins formed during roasting [28]. It has been suggested that the main antioxidant compounds present in the CSE are CGAs, melanoidins and antioxidant fiber. Such an antioxidant capacity that CSE possesses suggests that it could be used as a good source of bioactive compounds with putative health benefits [17].

Studies using different cell types, such as pancreatic cells, have demonstrated that CSE is not cytotoxic when used at the determined concentrations [26]. However, there are very few studies that report the effects of an aqueous CSE in the HaCaT cell line. Actually, Rodrigues and colleagues are the only ones who have previously studied the effect of CSE in HaCaT cells and fibroblasts, and CSE was not cytotoxic, as well [16,29]. Their studies on human skin cells involved aqueous, hydroalcoholic and ethanolic CSE in a final concentration range of 0.1–1000 μg/mL, the same concentrations used in our study. None of the extracts resulted in being cytotoxic in these cell lines [16].

Due to keratinocytes' location in the human body, these cells are continuously exposed to endogenous and environmental pro-oxidant agents, which increase intracellular levels of reactive oxygen species [30]. When skin is exposed to UV radiation, distinct response pathways are activated. As UV radiation causes the generation of ROS [31], we decided to induce cellular stress with *t*-BOOH. *tert*-Butyl hydroperoxide is a membrane-permeant oxidant that has been extensively used as a model of oxidative stress in different systems [32]. The range of *t*-BOOH doses used in our studies to induce cytotoxicity (0–1 mM) was similar to the one that Alía and colleagues used in HepG2 cells [33].

To investigate the potential of CSE in the protection against oxidative damage, we used effective doses of CSE on an *ex vivo* and on an *in vivo* model, HaCaT cells and *C. elegans*, respectively. The spontaneously immortalized human keratinocyte line, HaCaT, is one of the most frequently-used keratinocyte cell lines because of its highly preserved differentiation capacity [18]. HaCaT cells were pre-treated for 24 h to simulate a chronic use of CSE prior to oxidative damage. Our results suggest that this chronic application of CSE on human skin cells could prevent the effects produced by oxidative stress damage. Since 1 mg/mL of CSE prevented from cellular death induced by 0.5 mM *t*-BOOH and was able to reduce induced intracellular ROS to physiological levels (Figures 3–5), we could use this extract in this concentration to preserve skin health. Other studies have shown that CSE is able to protect pancreatic cells from induced oxidative damage [22]. Furthermore, there are other compounds present in coffee silverskin that can protect from UV-induced photodamage. On the one hand, research demonstrates that caffeine inhibited the development of squamous cell carcinomas when mice were previously treated with UV radiation. This suggests that caffeine is able to absorb as an additional sunscreen in the UV range and to prevent photodamage and photocarcinogenesis [34]. On the other hand, another study showed how *Coffea arabica* leaf extract and its constituents, chlorogenic acid and caffeic acid, diminished UV-induced photoaging by inhibiting MMPs through ROS scavenging and down-regulation of the MAP kinase pathway [12].

The prevention of UV damage is one of the most effective ways of diminishing the effects of photoaging, one of the biggest factors contributing to facial wrinkles [12]. The use of nematodes in this study is an interesting way of combining topical and oral administration of the bioactive compounds present in CSE. In fact, many studies suggest that using a combination of topical and oral antioxidants provides better results in the protection from UV radiation [21,35].

In the present study, we showed that UV-induced oxidative stress significantly decreased the viability and the lifespan of *C. elegans*. Furthermore, CSE restored the lifespan of oxidative stress-UV-induced *C. elegans*. It is well known that UV radiation is the main cause of photoaging and induces cell and tissue damage as the production of ROS, which leads to DNA damage [36]. In this sense, CSE could be reducing the oxidative stress accumulation and, therefore, the DNA damage, as previously demonstrated with other antioxidant compounds, such as tocotrienol [37]. Although previous reports have been made about the functional properties of coffee in *C. elegans* [38,39], we report for the first time the potential of a natural extract from coffee silverskin by-product for UV radiation protection, which could be very interesting for dermo- and nutria-cosmetic companies developing new products targeting photoaging. The chemical composition of coffee extracts studied by other authors is different from that corresponding to the coffee silverskin extract hereby investigated and patented by our research group.

Other studies use this nematode to study the effect of plant extracts on its lifespan. There are other plant extracts containing CGA and other polyphenols able to exert an antiaging effect on *C. elegans*. For instance, crude blueberry extract and blueberry polyphenols (including an hydroxycinnamic ester fraction containing CGA) have lengthened the nematode's mean lifespan by 28% [40]. Moreover, Vayndorf *et al.* observed that when *C. elegans* was pre-treated with whole apple extracts, worms were more resistant to stresses, such as heat, UV radiation and pathogenic infection, suggesting that cellular defense and immune system functions were improved. The authors suggest a possible antioxidant mechanism underlying the antiaging effects of whole apple phytochemicals [41]. In addition, polydatin, a natural resveratrol glycoside, was found to significantly extend the mean lifespan of worms by up to 30.7% and 62.1% under normal and heavy metal-induced acute stress conditions, respectively [42]. Some of these extracts have already shown their effectiveness as antiaging agents in humans [43], validating the feasibility of the animal model for the acquisition of preclinical data on the nutraceutical benefits of botanicals.

The antioxidant capacity of CSE is due to phenolic compounds, such as free chlorogenic acids and its derivatives, among others. Since in the cell culture model, neither CGA nor CAF at concentrations equivalent to those present in 1 mg/mL of CSE were able to prevent from oxidative damage, it seems that CGA and CAF are not solely responsible for the antioxidant capacity of CSE found under our particular experimental conditions. In fact, there are other antioxidant compounds present in the sample, such as melanoidins formed during roasting and antioxidant fiber, that may also contribute to such an effect [17,25]. Further research is needed to identify those compounds responsible for the CSE cellular antioxidant effect. Such a property may be due to a synergic effect derived from the combination of the bioactive compounds present in CSE.

The results obtained in the present study support the feasibility of using coffee silverskin extract in skin care for protection against skin diseases associated with oxidative stress and accelerated aging induced by UV radiation. The application of the extracts in cosmetology and dermatology represent an opportunity to increase the sustainability and competiveness of the coffee sector. The obtained data support that coffee is not only for drinking, in agreement with data reported by others indicating the feasibility of applying the biorefinery concept to the coffee sector [44].

Apart from roasting to prepare the coffee brew, the best known application for green coffee is as a natural source of antioxidants [45] and as weight-loss supplements [46]. Furthermore, *C. arabica* green coffee beans present a high content of oil, wax and unsaturated fatty acids, which leads to a high sun protection factor [47]. Coffee silverskin has also been suggested for use in cosmetic care products [15,16,48]. However, very little is known about the contribution of the individual components of the extracts to this effect and their mechanism of action. There is a lack of information regarding the chemical composition of the silverskin extract, although it is of great interest. Because of the accepted safety profile of these compounds, the addition of coffee extracts to sunscreen products could be considered [34]. Del Castillo *et al.* proposed the use of coffee silverskin in skin care cosmetics to prevent physiological aging in 2011 [15]. Last year, Rodrigues and colleagues studied a hand cream

formulation containing 2.5% (*w*/*w*) of CSE. Their studies confirm that it is possible to include CSE in a hand cream formulation and that such a product is stable under extreme conditions and safe for topical use [29].

Results support that the patented CSE (WO/2013/004873) feasibly reduces the production of intracellular ROS in keratinocytes, improving skin health. Additionally, CSE protects against photoaging induced by UV radiation.

4. Materials and Methods

4.1. Materials

Chlorogenic acid, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), caffeine, ascorbic acid (vitamin C), tert-butyl hydroperoxide (*t*-BOOH), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazole-y)-2,5-diphenyltetrazolium bromide (MTT) and 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical (Sigma-Aldrich, St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Lonza (Basel, Switzerland).

4.2. Preparation of Soluble Extracts from Coffee Silverskin

Arabica CSE was produced as described in the patent WO 2013/004873 [15]. Briefly, 50 mg of coffee silverskin were added per H_2O milliliter. This mixture was stirred at 250 rpm for 10 min; filtered by Whatman paper No. 4; and the filtrate was freeze-dried. Powdered CSE was prepared in aqueous solution, sterile filtered and added to medium to achieve final concentrations of 0.01 mg/mL, 0.1 mg/mL, 0.5 mg/mL and 1 mg/mL. CSE contained 19.87 $\pm$ 2.4 mg caffeine/g dry matter and 6.88 $\pm$ 1.77 mg CGA/g dry matter [22].

4.3. CSE Overall Antioxidant Capacity Assay

The trapping capacity of cationic free radicals was evaluated using the method of radical $ABTS^{\bullet+}$ bleaching described by Re *et al.* 1999 [49] and modified by Oki *et al.* [50] for its use in a microplate. A stock solution of the $ABTS^{\bullet+}$ radical was prepared by chemical oxidation of ABTS (7 mM) in the presence of potassium persulfate (140 mM) at room temperature and in darkness for 16 h. The working solution of the $ABTS^{\bullet+}$ radical was prepared by diluting the stock solution 1:75 (*v*/*v*) in 5 mM sodium phosphate buffer (pH 7.4) to obtain an absorbance value of 0.7 $\pm$ 0.02 at 734 nm. Since CGA is the major antioxidant component in coffee, CGA calibration was used to calculate overall antioxidant capacity. A 1:10 dilution (*v*/*v*) of the CGA pattern was performed, so that the final concentrations of the CGA pattern used were 11.5 µM, 25 µM, 50 µM, 75 µM, 115 µM and 200 µM. Then, 30 µL of the samples and 270 µL of the working solution of $ABTS^{\bullet+}$ radical were placed in a microplate (Microtest PS plate 96, Sarstedt AG & Co, Nümbrecht, Germany), and absorbance was measured at 734 nm after 10 min in a BioTek plate reader powerWave™ XS (BioTek Instruments, Winooski, VT, USA).

All determinations were carried out in triplicate. Absorbance values were corrected for the solvent, and inhibition percentages were obtained by multiplying the values of ΔA_{sample} by 100.

4.4. Cell Culture and Treatments

The HaCaT human keratinocyte cell line was kindly provided by Dr. Miguel Quintanilla (Instituto de Investigaciones Biomédicas "Alberto Sols", Madrid, Spain). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin in standard conditions (37 °C, 5% CO_2, in a humidified incubator, BINDER CB series 2010, Tuttlingen, Germany).

For the treatments with the different compounds, concentrations of CSE (0.01, 0.1, 0.5 and 1 mg/mL), CGA (6.88 µg/mL), caffeine (19.86 µg/mL) and vitamin C (0.1 µg/mL) diluted in DMEM culture medium and filtered through a 0.45-µm membrane were added to cell plates during 24 h. In

order to induce oxidative stress in cells, *t*-BOOH was dissolved in MilliQ· H_2O and added to cell plates during different periods of time (1, 6 and 24 h) and at concentrations ranging from 0.1–1 mM.

4.5. Cell Viability Assays

The effect of different concentrations of CSE, chlorogenic acid, caffeine and vitamin C alone or in combination with *t*-BOOH on cell viability was measured using the MTT assay [51]. Cells were cultured at a density of 1.0×10^4 cells per well of a 96-well plate for 3 days until cell confluence was achieved. On the one hand, CSE (0.01 mg/mL, 0.1 mg/mL, 0.5 mg/mL and 1 mg/mL), chlorogenic acid (6.88 µg/mL), caffeine (19.86 µg/mL) and vitamin C (0.1 µg/mL) were incubated for 24 h. Triton X-100 (10%) was used as the death control. On the other hand, different concentrations of *t*-BOOH (0.1 mM, 0.25 mM 0.5 mM and 1 mM) were studied for 1 h, 6 h and 24 h. Subsequently, cells were incubated in MTT solution (0.5 mg/mL) for 1 h at 37 °C. The supernatant was then removed; 100 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, Tres Cantos, Madrid, Spain) were added; and the optical density at 570 nm was measured using a microplate reader (BioTek Synergy HT Multi-Mode Microplate Reader, Winooski, VT, USA). Experiments were carried out in triplicate ($n = 6$).

4.6. ROS Scavenging Assay

Intracellular ROS scavenging assays were performed by measuring the fluorescence intensity of the 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe, which was proportional to the amount of ROS formed [52]. A 10 mM solution of DCFH-DA was prepared (5 mg in 1 mL DMSO), and a 50-µL aliquot was separated. Then, 800 µL of DMSO were added to the 50 µL solution. Next, after 24 h of extract incubation, cells were pre-loaded with 2.5 µL/well of this last solution for 30 min at 37 °C. After incubation, DCFH will become dichlorofluorescein (DCF) due to intracellular oxidants and will emit fluorescence. Next, the culture medium was removed; cells were washed with PBS; and *t*-BOOH was added for 1 h. Then, fluorescence was measured at 485 nm/528 nm (BioTek Synergy HT Multi-Mode Microplate Reader). Experiments were carried out in triplicate ($n = 6$).

4.7. C. elegans Lifespan Assays

To measure *C. elegans* survival rates after exposure to oxidative stress induced by UVC radiation, we employed synchronized *C. elegans* N2 strain eggs. They hatched in NG medium (nematode growth) and were cultured on agar plates containing *Escherichia coli* OP50 strain. After 3 days of growth at 20 °C, worms were transferred to plates containing NG medium, *E. coli* and different concentrations of CSE (0.01 mg/mL, 0.1 mg/mL and 1 mg/mL), CGA (0.1 µg/mL) or vitamin C (0.1 µg/mL). Then, worms (100 worms per treatment) were incubated for 15 days at 20 °C and transferred every 2 days to fresh media plates to score viability. During this period, worms were subjected daily to UVC radiation for 45 s. The animals were scored as dead if they failed to respond to a platinum wire. All assays were performed in triplicate.

4.8. Statistical Analyses

Data obtained from *ex vivo* experiments were expressed as the mean ± SD of 18 determinations. One-way analysis of variance (ANOVA) was performed for cytotoxicity and ROS analysis in HaCaT cells. Statistical comparisons of the different treatments were performed using Tukey's test. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using the R package software environment Version 3.2.0 (http://www.r-project.org/).

Survival curves of the cultured nematodes in the presence of CSE, CGA or vitamin C were plotted and analyzed using GraphPad Prism 4 (http://www.graphpad.com/scientific-software/prism/) to study the significance in the viability increase of *C. elegans* among the different conditions. Values of $p < 0.05$ were considered statistically significant.

5. Conclusions

We provide scientific evidence with regard to the antioxidant protective effects of CSE in human skin cells and *in vivo* using *C. elegans*, an experimental model. Pure CGA and CAF at the concentrations equivalent to those present in 1 mg/mL of CSE do not seem to be effective in the protection of HaCaT cells from oxidative damage, so further experiments should be performed in order to determine their contribution to the overall antioxidant effect of the extract. CSE is a complex mixture of antioxidants, including CGA, melanoidins and others. Therefore, the protective effect of CSE may be due to the synergistic combination of individual compounds, including phenols, such as CGA. Additional investigation should be carried out to identify all of the antioxidants forming the food matrix. In conclusion, it can be said that CSE has the potential to be used as an ingredient in skin care products for topical use and as nutricosmetic to prevent accelerated skin aging induced by oxidative stress caused either by chemical of physical agents (photoaging).

Supplementary Materials: Supplementary materials can be accessed at: http://www.mdpi.com/1420-3049/21/6/721/s1.

Acknowledgments: The authors are grateful for the financial support from the SUSCOFFEE Project (AGL2014-57239-R) and the NATURAGE Project (AGL2010-17779). This work was partially funded by a Santander Small and Medium Enterprises Work Placement Grant in Beacon Biomedicine. Amaia Iriondo is a fellow of the FPI predoctoral program of the Ministry of Economy and Competitiveness (BES-2015-072191). Konstantinos Stamatakis is a recipient of an Asociación Española Contra el Cancer fellowship.

Author Contributions: Amaia Iriondo-DeHond is the first author of the paper. Part of the results described in the present paper belongs to her MSc thesis. Amaia Iriondo-DeHond, Konstantinos Stamatakis and Manuel Fresno were involved in the experiments performed in HaCaT cells. Patricia Martorell, Salvador Genovés and Daniel Ramón conducted the experiments on *C. elegans*. Antonio Molina was involved in the experimental design of the research carried out in cells. María Dolores del Castillo was the principal investigator of the research and supervisor of Amaia's job.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hancock, J.T.; Desikan, R.; Neill, S.J. Role of reactive oxygen species in cell signalling pathways. *Biochem. Soc. Trans.* **2001**, *29*, 345–350. [CrossRef] [PubMed]
2. Klaunig, J.E.; Kamendulis, L.M.; Hocevar, B. A Oxidative stress and oxidative damage in carcinogenesis. *Toxicol. Pathol.* **2010**, *38*, 96–109. [CrossRef] [PubMed]
3. Kruk, J.; Duchnik, E. Oxidative Stress and Skin Diseases: Possible Role of Physical Activity. *Asian Pac. J. Cancer Prev.* **2014**, *15*, 561–568. [CrossRef] [PubMed]
4. Dalle-Donne, I.; Rossi, R.; Colombo, R.; Giustarini, D.; Milzani, A. Biomarkers of oxidative damage in human disease. *Clin. Chem.* **2006**, *52*, 601–623. [CrossRef] [PubMed]
5. Garigan, D.; Hsu, A.L.; Fraser, A.G.; Kamath, R.S.; Abringet, J.; Kenyon, C. Genetic analysis of tissue aging in *Caenorhabditis elegans*: A role for heat-shock factor and bacterial proliferation. *Genetics* **2002**, *161*, 1101–1112. [PubMed]
6. Bisht, S.; Sisodia, S. *Coffea arabica*: A wonder gift to medical science. *J. Nat. Pharm.* **2010**, *1*, 58–65. [CrossRef]
7. Fava, F.; Totaro, G.; Diels, L.; Reis, M.; Duarte, J.; Carioca, O.B.; Poggi-Varaldo, H.M.; Ferreira, B.S. Biowaste biorefinery in Europe: Opportunities and research & development needs. *New Biotechnol.* **2015**, *32*, 100–108.
8. Park, K.; Lee, J.H. Protective effects of resveratrol on UVB-irradiated HaCaT cells through attenuation of the caspase pathway. *Oncol. Rep.* **2008**, *19*, 413–417. [CrossRef] [PubMed]
9. Vitale, N.; Kisslinger, A.; Paladino, S.; Procaccini, C.; Matarese, G.; Pierantoni, G.M.; Mancini, F.P.; Tramontano, D. Resveratrol couples apoptosis with autophagy in UVB-irradiated HaCaT cells. *PLoS ONE* **2013**, *8*, e80728. [CrossRef] [PubMed]
10. Pérez-Sánchez, A.; Barrajón-Catalán, E.; Caturla, N.; Castillo, J.; Benavente-García, O.; Alcaraz, M.; Micol, V. Protective effects of citrus and rosemary extracts on UV-induced damage in skin cell model and human volunteers. *J. Photochem. Photobiol. B Biol.* **2014**, *136*, 12–18. [CrossRef] [PubMed]

11. Almeida, I.F.; Pinto, A.S.; Monteiro, C.; Monteiro, H.; Belo, L.; Fernandes, J.; Bento, A.R.; Duarte, T.L.; Garrido, J.; Bahia, M.F.; *et al.* Protective effect of C. sativa leaf extract against UV mediated-DNA damage in a human keratinocyte cell line. *J. Photochem. Photobiol. B Biol.* **2015**, *144*, 28–34. [CrossRef] [PubMed]
12. Chiang, H.M.; Lin, T.J.; Chiu, C.Y.; Chang, C.W.; Hsu, K.C.; Fan, P.C.; Wen, K.C. Coffea arabica extract and its constituents prevent photoaging by suppressing MMPs expression and MAP kinase pathway. *Food Chem. Toxicol.* **2011**, *49*, 309–318. [CrossRef] [PubMed]
13. Herman, A.; Herman, A.P. Caffeine's mechanisms of action and its cosmetic use. *Skin Pharmacol. Physiol.* **2012**, *26*, 8–14. [CrossRef] [PubMed]
14. Fukushima, Y.; Takahashi, Y.; Hori, Y.; Kishimoto, Y.; Shiga, K.; Tanaka, Y.; Masunaga, E.; Tani, M.; Yokoyama, M.; Kondo, K. Skin photoprotection and consumption of coffee and polyphenols in healthy middle-aged Japanese females. *Int. J. Dermatol.* **2015**, *54*, 410–418. [CrossRef] [PubMed]
15. Del Castillo, B.M.D.; Ibañez, E.M.E.; Amigo, B.M.; Herrero, C.M.; PLaza, del M.M.; Ullate, A.M. Application of Products of Coffee Silverskin in Anti-agenic Cosmetic and Functional Food. Patent EP2730171 A1, 14 May 2014.
16. Rodrigues, F.; Palmeira-de-Oliveira, A.; das Neves, J.; Sarmento, B.; Amaral, M.H.; Oliveira, M.B.P.P. Coffee silverskin: A possible valuable cosmetic ingredient. *Pharm. Biol.* **2015**, *53*, 386–394. [CrossRef] [PubMed]
17. Del Castillo, M.D.; Fernandez-Gomez, B.; Martinez-Saez, N.; Iriondo-DeHond, A.; Mesa, M.D. Coffee by-products. In *Coffee: Chemistry, Quality and Health Implications*; Farah, A., Ed.; RSC Publishing Inc: Cambridge, UK, In press.
18. Brohem, C.A.; Da Silva Cardeal, L.B.; Tiago, M.; Soengas, M.S.; De Moraes Barros, S.B.; Maria-Engler, S.S. Artificial skin in perspective: Concepts and applications. *Pigment Cell Melanoma Res.* **2011**, *24*, 35–50. [CrossRef] [PubMed]
19. Mazzoleni, G.; di Lorenzo, D.; Steimberg, N. Modelling tissues in 3D: The next future of pharmaco-toxicology and food research? *Genes Nutr.* **2009**, *4*, 13–22. [CrossRef] [PubMed]
20. Kenyon, C. The plasticity of aging: Insights from long-lived mutants. *Cell* **2005**, *120*, 449–460. [CrossRef] [PubMed]
21. Baumann, L. Skin ageing and its treatment. *J. Pathol.* **2007**, *211*, 241–251. [CrossRef] [PubMed]
22. Fernandez-Gomez, B.; Nitride, C.; Ullate, M.; Mamone, G.; Ferranti, P.; del Castillo, M.D. Use of phytochemomics for validating the potential of coffee silverskin extract as natural source of inhibitors of the glycoxidation reaction. *Food Funct.* **2016**. underprocessing.
23. Kucera, O.; Endlicher, R.; Rousar, T.; Lotková, H.; Garnol, T.; Drahota, Z.; Cervinková, Z. The effect of tert -butyl hydroperoxide-induced oxidative stress on lean and steatotic rat hepatocytes *in vitro*. *Oxid. Med. Cell. Longev.* **2014**, *2014*, 752506. [CrossRef] [PubMed]
24. Boyd, W.A.; Crocker, T.L.; Rodriguez, A.M.; Leung, M.C.K.; Wade, D.; Freedman, J.H.; Van Houten, B.; Meyer, J.N. Nucleotide excision repair genes are expressed at low levels and are not detectably inducible in *Caenorhabditis elegans* somatic tissues, but their function is required for normal adult life after UVC exposure. *Mutat. Res.* **2010**, *683*, 57–67. [CrossRef] [PubMed]
25. Mesías, M.; Navarro, M.; Martínez-Saez, N.; Ullate, M.; del Castillo, M.D.; Morales, F.J. Antiglycative and carbonyl trapping properties of the water soluble fraction of coffee silverskin. *Food Res. Int.* **2014**, *62*, 1120–1126. [CrossRef]
26. Fernandez-Gomez, B.; Ramos, S.; Goya, L.; Mesa, M.D.; del Castillo, M.D.; Martín, M.Á. Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E β cells. *Food Res. Int.* **2016**. in press. [CrossRef]
27. Narita, Y.; Inouye, K. High antioxidant activity of coffee silverskin extracts obtained by the treatment of coffee silverskin with subcritical water. *Food Chem.* **2012**, *135*, 943–949. [CrossRef] [PubMed]
28. Borrelli, R.C.; Esposito, F.; Napolitano, A.; Ritieni, A.; Fogliano, V. Characterization of a new potential functional ingredient: Coffee silverskin. *J. Agric. Food Chem.* **2004**, *52*, 1338–1343. [CrossRef] [PubMed]
29. Rodrigues, F.; Gaspar, C.; Palmeira-de-Oliveira, A.; Sarmento, B.; Helena Amaral, M.; P P Oliveira, M.B. Application of Coffee Silverskin in cosmetic formulations: Physical/antioxidant stability studies and cytotoxicity effects. *Drug Dev. Ind. Pharm.* **2015**, 1–8. [CrossRef] [PubMed]
30. Bae, S.; Lee, E.-J.; Lee, J.H.; Park, I.-C.; Lee, S.-J.; Hahn, H.J.; Ahn, H.J.; An, S.; An, I.-S.; Cha, H.J. Oridonin protects HaCaT keratinocytes against hydrogen peroxide-induced oxidative stress by altering microRNA expression. *Int. J. Mol. Med.* **2013**, 185–193. [CrossRef]

31. Birch-Machin, M.A.; Swalwell, H. How mitochondria record the effects of UV exposure and oxidative stress using human skin as a model tissue. *Mutagenesis* **2010**, *25*, 101–107. [CrossRef] [PubMed]
32. Garcia-Cohen, E.C.; Marin, J.; Diez-Picazo, L.D.; Baena, A.B.; Salaices, M.; Rodriguez-Martinez, M.A. Oxidative stress induced by *tert*-butyl hydroperoxide causes vasoconstriction in the aorta from hypertensive and aged rats: Role of cyclooxygenase-2 isoform. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 75–81. [PubMed]
33. Alía, M.; Ramos, S.; Mateos, R.; Bravo, L.; Goya, L. Response of the antioxidant defense system to *tert*-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *J. Biochem. Mol. Toxicol.* **2005**, *19*, 119–128. [CrossRef] [PubMed]
34. Koo, S.W.; Hirakawa, S.; Fujii, S.; Kawasumi, M.; Nghiem, P. Protection from photodamage by topical application of caffeine after ultraviolet irradiation. *Br. J. Dermatol.* **2007**, *156*, 957–964. [CrossRef] [PubMed]
35. Fernández-García, E. Skin protection against UV light by dietary antioxidants. *Food Funct.* **2014**, *5*, 1994–2003. [CrossRef] [PubMed]
36. Pandel, R.; Poljšak, B.; Godic, A.; Dahmane, R. Skin Photoaging and the Role of Antioxidants in Its Prevention. *ISRN Dermatol.* **2013**, *2013*. [CrossRef] [PubMed]
37. Aan, G.J.; Zainudin, M.S.A.; Karim, N.A.; Ngah, W.Z.W. Effect of the tocotrienol-rich fraction on the lifespan and oxidative biomarkers in *Caenorhabditis elegans* under oxidative stress. *Clinics (Sao Paulo)* **2013**, *68*, 599–604. [CrossRef]
38. Dostal, V.; Roberts, C.M.; Link, C.D. Genetic mechanisms of coffee extract protection in a *Caenorhabditis elegans* model of β-amyloid peptide toxicity. *Genetics* **2010**, *186*, 857–866. [CrossRef] [PubMed]
39. Fitzenberger, E.; Deusing, D.J.; Wittkop, A.; Kler, A.; Kriesl, E.; Bonnländer, B.; Wenzel, U. Effects of Plant Extracts on the Reversal of Glucose-Induced Impairment of Stress-Resistance in *Caenorhabditis elegans*. *Plant Foods Hum. Nutr.* **2014**, *69*, 78–84. [CrossRef] [PubMed]
40. Wilson, M.A.; Shukitt-Hale, B.; Kalt, W.; Ingram, D.K.; Joseph, J.A.; Wolkow, C.A. Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*. *Aging Cell* **2006**, *5*, 59–68. [CrossRef] [PubMed]
41. Vayndorf, E.M.; Lee, S.S.; Liu, R.H. Whole apple extracts increase lifespan, healthspan and resistance to stress in *Caenorhabditis elegans*. *J. Funct. Foods* **2013**, *5*, 1235–1243. [CrossRef] [PubMed]
42. Wen, H.; Gao, X.; Qin, J. Probing the anti-aging role of polydatin in *Caenorhabditis elegans* on a chip. *Integr. Biol. (Camb.)* **2013**, *6*, 35–43. [CrossRef] [PubMed]
43. Jadoon, S.; Karim, S.; Asad, M.H.H.B.; Akram, M.R.; Kalsoom Khan, A.; Malik, A.; Chen, C.; Murtaza, G. Anti-aging potential of phytoextract loaded-pharmaceutical creams for human skin cell longetivity. *Oxid. Med. Cell. Longev.* **2015**, *2015*. [CrossRef] [PubMed]
44. Choplet, M.; Annevelink, B. Biorefinery Euroview. Current Situation and Potential of the Biorefinery concept in the EU: Strategic Framework and Guidelines for Its Development. Avaliable online: http://www.biorefinery.nl/fileadmin/biopol/user/documents/PublicDeliverables/BIOPOL_D_4_2_PUBLIC_with_EUROVIEW_-_Final_100709_opt.pdf (accessed on 15 December 2015).
45. Fukushima, Y.; Ohie, T.; Yonekawa, Y.; Yonemoto, K.; Aizawa, H.; Mori, Y.; Watanabe, M.; Takeuchi, M.; Hasegawa, M.; Taguchi, C.; *et al.* Coffee and green tea as a large source of antioxidant polyphenols in the Japanese population. *J. Agric. Food Chem.* **2009**, *57*, 1253–1259. [CrossRef] [PubMed]
46. Onakpoya, I.; Terry, R.; Ernst, E. The use of green coffee extract as a weight loss supplement: A systematic review and meta-analysis of randomised clinical trials. *Gastroenterol. Res. Pract.* **2011**, *2011*. [CrossRef] [PubMed]
47. Wagemaker, T.A.L.; Carvalho, C.R.L.; Maia, N.B.; Baggio, S.R.; Guerreiro Filho, O. Sun protection factor, content and composition of lipid fraction of green coffee beans. *Ind. Crops Prod.* **2011**, *33*, 469–473. [CrossRef]
48. Rodrigues, F.; Pereira, C.; Pimentel, F.B.; Alves, R.C.; Ferreira, M.; Sarmento, B.; Amaral, M.H.; Oliveira, M.B.P.P. Are coffee silverskin extracts safe for topical use? An *in vitro* and *in vivo* approach. *Ind. Crops Prod.* **2015**, *63*, 167–174. [CrossRef]
49. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
50. Oki, T.; Nagai, S.; Yoshinaga, M.; Nishiba, Y.; Suda, I. Contribution of β-Carotene to Radical Scavenging Capacity Varies among Orange-fleshed Sweet Potato Cultivars. *Food Sci. Technol. Res* **2006**, *12*, 156–160. [CrossRef]

51. Bakondi, E.; Gönczi, M.; Szabó, É.; Bai, P.; Pacher, P.; Gergely, P.; Kovács, L.; Hunyadi, J.; Szabó, C.; Csernoch, L.; *et al.* Role of intracellular calcium mobilization and cell-density-dependent signaling in oxidative-stress-induced cytotoxicity in HaCaT keratinocytes. *J. Investig. Dermatol.* **2003**, *121*, 88–95. [CrossRef] [PubMed]
52. Gomes, A.; Fernandes, E.; Lima, J.L.F.C. Fluorescence probes used for detection of reactive oxygen species. *J. Biochem. Biophys. Methods* **2005**, *65*, 45–80. [CrossRef] [PubMed]

Sample Availability: Samples of CSE are available from the authors for official collaboration.

Molecules **2016**, *21*, 721; doi:10.3390/molecules21060721

Supplementary Materials: Coffee Silverskin Extract Protects Against Accelerated Aging Caused by Oxidative Agents

Amaia Iriondo-DeHond, Patricia Martorell, Salvador Genovés, Daniel Ramón, Konstantinos Stamatakis, Manuel Fresno, Antonio Molina and María Dolores del Castillo

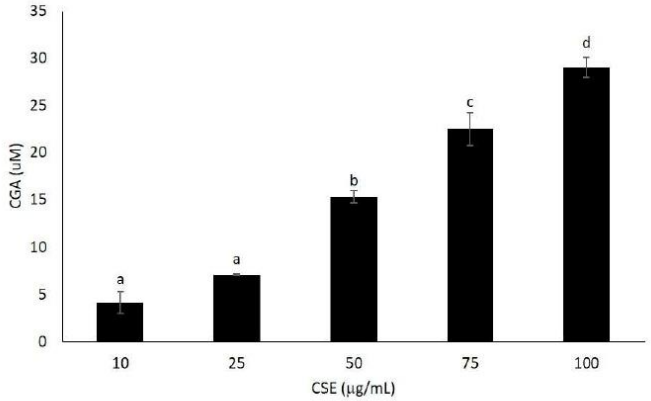


Figure S1. Antioxidant capacity of CSE determined by the ABTS$^{\bullet+}$ assay represented in CGA equivalents (µM). Samples with different letter differ significantly (Tukey Test. $p < 0.05$).

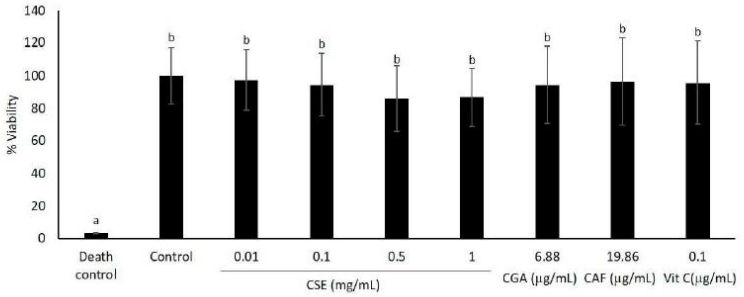


Figure S2. Cytotoxicity assessment of CSE (0.01 mg/mL, 0.1 mg/mL, 0.5 mg/mL and 1 mg/mL), CGA (6.88 µg/mL), caffeine (19.86 µg/mL) and vitamin C (0.1 µg/mL) determined by the MTT method. Triton X-100 (10%) was used as the death control. Results represent the percentage of viable cells after 24 hours of incubation with the different compounds. Data are expressed as the mean of 18 replicates ± SD. Treatments with different letters differ significantly (Tukey Test, $p < 0.05$).

Personal Care Products Council
Committed to Safety, Quality & Innovation

October 20, 2017

Mr. Juan Perez Cano
Santiago Grisolia 2
Tres Cantos, 28760
Kingdom of Spain

RE: Application No. 2-03-2017-6117

Dear Mr. Juan Perez Cano:

In reference to the INCI application noted above, the International Cosmetic Ingredient Nomenclature Committee (INC) has completed its review of your request. The INCI name assigned to the trade name identified in this application is detailed on the attachment.

Please note, the attachment lists information from your application as it appears in our data base and will be published in the *International Cosmetic Ingredient Dictionary and Handbook,* the web-based Dictionary *wINCI,* and the Council's *On-Line INFOBASE*. If your application indicates the trade name is "not for publication," it is noted on the attachment and the data will not be published.

It is important to carefully check the attachment for accuracy and respond to our office promptly with any changes. The INCI name assignment and related company information will be retained in our data base unless we are notified that the product is no longer manufactured. You will be contacted on an approximate annual basis to update the current status of your company listings. This communication will include only trade names and addresses; therefore it is imperative that you maintain accurate records of all INCI name assignments.

To petition for a change in an INCI name assignment, a request to the INC can be sent via email to my attention. The petition should include the current INCI name, trade name, application number, requested revision, and technical rationale to support the petition, e.g., supporting composition information, and/or manufacturing details, and analytical data where appropriate.

In addition, please be advised that INCI names are continually reviewed by the INC for accuracy, and may be subject to change when deemed necessary.

Should you have any questions, please don't hesitate to contact me for further information.

Sincerely,

Joanne Nikitakis

Joanne M. Nikitakis
Director, Cosmetic Chemistry
nikitakisj@personalcarecouncil.org
Enclosure

Personal Care Products Council
Committed to Safety, Quality & Innovation

October 20, 2017

Application No. 2-03-2017-6117

Submitted By:
Mr. Juan Perez Cano
Santiago Grisolia 2
Tres Cantos, 28760
Kingdom of Spain

Manufactured By:
Beacon Biomedicine S.L.
Santiago de Grisolía 2
Tres Cantos
Madrid, Madrid, 28760
SPAIN

Trade Name:
SILVERSKIN

Assigned INCI Name:
Water (and) Coffea Arabica/Robusta Chaff Extract

CURRICULUM VITAE

Education

- **Doctoral program** in Food Sciences (2015-2019). Universidad Autónoma de Madrid (UAM), Spain.
- **Master's degree** in in Food Engineering Applied to Health (2014-2015), Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid (UPM), Spain.
- **Bachelor's degree** in Biochemistry (2010-2014), Universidad Autónoma de Madrid, Spain.

Fellowships and research experience

- **Pre-doctoral Student Fellowship** (2015 - 2019) – funded by the Ministry of Economy and Competitiveness of Spain (BES-2015-072191). "Validation of coffee by-products as food ingredients for sustainable health and nutrition" at the Instituto de Investigación en Ciencias de la Alimentación (CIAL), Consejo Superior de Investigaciones Científicas (CSIC). Madrid, Spain.

- **Visiting PhD Student** (2017 February – May) – "Study of the effect of spent coffee grounds in gastrointestinal health in rats" at the Pharmacology Group from the Departamento de Ciencias Básicas de la Salud of the Universidad Rey Juan Carlos (URJC), Madrid, Spain. P.I. Dr. Raquel Abalo.

- **Visiting PhD Student** (September 2016 – January 2017) - "New knowledge on the therapeutic effect of coffee silverskin extract in chronic metabolic disorders" at the Nutrition/Metabolism Laboratory at Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School. Boston, MA, USA. P.I. Dr. Jin-Rong Zhou.

- **Visiting PhD Student** (2016 February – June) – Group of Estrategias quimiopreventivas frentes a compuestos carcinogénicos de la dieta, Facultad de Veterinaria, Universidad Complutense de Madrid (UCM), Spain. (https://filologia.ucm.es/grupos/grupo/24) P.I. Dr. Paloma Morales.

- **Research Student Grant** (2015 June-September) - Santander Small and Medium Enterprise Work Placement Grants in Beacon Biomedicine. Madrid, Spain.

- **Master Thesis** (2015 January – July) "Sustainable use of coffee extracts in skin health" at Instituto de Investigación en Ciencias de la Alimentación (CIAL, UAM-CSIC), Madrid, Spain. Supervised by Dr. María Dolores del Castillo and Dr. Konstantinos Stamatakis.

- **Student Grant** (2015 January – July), Innovative Education Project. Intern in the Physics Department, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid (UPM). Madrid, Spain.

- **Technical English translator** (2014 July – October) for the labeling of Despemsa products (DESPEMSA UNION S.A.) sold in the European Union. Madrid, Spain.

- **Bachelor Thesis** (2013 –2014) "Study of generation and characterization of induced pluripotent stem cells by non-viral vectors" at Neural Regeneration Unit, Instituto de Salud Carlos III (ISCIII), Majadahonda, Madrid, Spain. Supervised by Dr. Isabel Liste Noya.

Participation in research projects

- FROM WASTE TO WORTH, EIT FOOD. (2019). Participation: collaborator from CIAL-CSIC.
- SUSCOFFEE, AGL2014-57239-R, MINECO. "Producción y consumo sostenibles del café: validación de subproductos como ingredientes alimentarios" (2015 - 2018). Participation: collaborator from CIAL-CSIC.
- METASIN, CIEN, CDTI. "Investigación, desarrollo e innovación en nuevos alimentos multifuncionales para síndrome metabólico" (2015 – 2018). Participation: collaborator from CIAL-CSIC.
- ALIBIRD-CM, S2013/ABI-2728, Comunidad Autónoma de Madrid. "Alimentos funcionales y estrategias nutricionales eficaces para la prevención y tratamiento de enfermedades crónicas" (2014 – 2018). Participation: collaborator from CIAL-CSIC.

Publications

Publications derived from PhD Thesis: (see "*List of publications*" section for publication details).

- Research articles: published (8), submitted (1).
- Book chapters: published (5), in press (1).
- Participation in conferences: 20 (6 oral communications and 14 posters).

Other publications:

- T. Herrera, **A. Iriondo-DeHond**, J. Uribarri, M.D. del Castillo. (2020). Beneficial herbs and spices. In *Nutrition, Fitness, and Mindfulness: An Evidence-Based Guide for Clinicians.* Edited by J. Uribarri and J.A. Vassalotti, Springer International Publishing AG.
- Revilla, A., González, C., **Iriondo, A**., Fernández, B., Prieto, C., Marín, C., Liste, I. 2015. Current advances in generation of human iPS cells: implications in cell-based regenerative medicine. *Journal of Tissue Engineering and Regenerative Medicine*. DOI:10.1002/term.2021.

Professional affiliations and membership

- (2019 – Present) American Society for Nutrition.
- (2019 – Present) Sociedad Española de Nutrición, Spain.
- (2017 – Present) AlimentUS. Cofounder and secretary of association.

Participation in scientific conferences

1. A.M. Fernández-Fernández, **A. Iriondo-DeHond**, E. Dellacassa, A. Medrano-Fernandez, & M.D. del Castillo. "Valorization of Tannat grape skin into health promoting food ingredients." Congreso CIBIA, 1st – 4th July 2019. Faro-Algarve, Portugal. Oral presentation.
2. **A. Iriondo-DeHond**, A. Rodríguez-Bertos, F. Nuñez, M.I. San Andres, S. Sanchez-Fortun, M.D. del Castillo. "Effect of the repeated intake of coffee silverskin extract on the short-chain fatty acid profile of rat feces." Fifth International Conference on Cocoa, coffee and tea (Cocotea) 26th - 28th June 2019. Bremen, Germany. Poster.
3. **A. Iriondo-DeHond**. "Introducción general a los co-productos del cultivo del café". Avances en la Ciencia y Tecnología del Café II, 15th – 17th May 2019. Bogotá Colombia. Oral presentation.
4. **A. Iriondo-DeHond**. "Validación de co-productos del cultivo de café como ingredientes alimentarios". Avances en la Ciencia y Tecnología del Café II, 15th – 17th May 2019. Bogotá Colombia. Oral presentation.
5. M.B. Ríos, **A. Iriondo-DeHond**, M. Iriondo-DeHond, M. Spaggiari, D. Arias, M.J. Callejo, M.D. del Castillo. "Coffee cascara gluten-free bread: a healthy lipid profile?". XXIII Jornadas de Nutrición Práctica, 3rd – 4th April 2019. Madrid, Spain. Poster.
6. S. Tores De La Cruz, **A. Iriondo-DeHond**, T. Herrera, Y. Lopez-Tofiño, R. Abalo, M.D. del Castillo. "Aislamiento de melanoidinas de cascarilla de café y evaluación de su bioactividad in vitro e in vivo." III Jornadas Científicas CIAL Fórum. 22nd – 23rd November 2018. Instituto de Investigación en Ciencias de la Alimentación (CIAL, UAM-CSIC), Madrid, Spain. Oral presentation.
7. M. Iriondo-DeHond, **A. Iriondo-DeHond**, T. Herrera, C.O. Sorzano, E. Miguel, M.D. del Castillo. "Randomized crossover nutritional trial of yogurts containing dietary-fiber and coffee cascara extract in healthy adults". XII Congreso de la Sociedad Española de Nutrición Comunitaria (SENC), IV World Congress of Public Health and Nutrition, 24th – 27th October 2018. Madrid, Spain. Poster.
8. **A. Iriondo-DeHond**, B. Ramírez, F. Velázquez Escobar, M.D. del Castillo. "Characterization and bioactivity of enriched fractions of melanoidins obtained from coffee by-products". XII Congreso de la Sociedad Española de Nutrición

Comunitaria (SENC), IV World Congress of Public Health and Nutrition, 24th – 27th October 2018. Madrid, Spain. Poster.

9. **A. Iriondo-DeHond**, N. Aparicio García, B. Fernandez-Gomez, E. Guisantes Batan, F. Velazquez Escobar, G.P. Blanch, M.I. San Andres, S. Sanchez-Fortun, M.D. del Castillo. "Food waste recovery for health sustainability. Coffee case." 5th International ISEKI_Food Conference, 3rd - 5th July 2018. Stuttgart, Germany. Oral presentation.
10. **A. Iriondo-DeHond**, S. Sanchez-Fortun, M.I. San Andres, M.D. del Castillo. "Data for the validation of coffee silverskin extract as a novel food ingredient". 5th International ISEKI_Food Conference, 3rd - 5th July 2018. Stuttgart, Germany. Poster.
11. **A. Iriondo-DeHond**, H.M. Abdolmaleky, J.R. Zhou, M.D. del Castillo. "Assessment of antioxidant and anti-inflammatory effects of coffee by-product extracts in a cellular model of diabetic nephropathy". XXII Jornadas de Nutrición Práctica, 11th – 12th April 2018. Madrid, Spain. Poster.
12. M. Iriondo-DeHond, J.M. Blázquez, M. Guzmán, **A. Iriondo-DeHond**, M. D. del Castillo, E. Miguel. "Sustainable functional yogurts for chronic disease prevention" 31st EFFoST International Conference, 13th – 16th November 2017. Barcelona, Spain. Poster.
13. R. Abalo, G. Vera, **A. Iriondo-DeHond**, L. Lopez-Gomez, J.A. Uranga, M.D. del Castillo. "Spent coffee grounds activate intestinal motility and are safe upon chronic treatment. Radiographic and histological study in rats". 22th International Conference of FFC - 10th International Symposium of ASFFBC: "Functional and Medical Foods for Chronic Diseases: Bioactive Compounds and Biomarkers". 22nd -23th September 2017. The Joseph B. Martin Conference Center at Harvard Medical School, Boston, USA. Poster.
14. M.D. del Castillo, F. Santillan-Cornejo, B. Fernandez-Gomez, **A. Iriondo-DeHond**, G. Vera, R. Abalo. "Lipid biomarkers for validation of spent coffee grounds as a healthy dietary fiber". 22th International Conference of FFC - 10th International Symposium of ASFFBC: "Functional and Medical Foods for Chronic Diseases: Bioactive Compounds and Biomarkers". 22nd -23th September 2017. The Joseph B. Martin Conference Center at Harvard Medical School, Boston, USA. Oral presentation.
15. M.D. del Castillo**, A. Iriondo-DeHond**, N. Martinez-Saez, B. Fernández-Gómez, M, Iriondo-DeHond, F. Velazquez-Escobar, J.R, Zhou. "Applications of recovered compounds in food products". Fourth International Conference on

16. Cocoa Coffee and Tea. 25th - 28th June 2017. Centro Congressi Torino Incontra, Turin, Italy. Oral presentation.
17. M. Iriondo-DeHond, **A. Iriondo-DeHond**, B. Fernández-Gómez, E. Miguel, M. D. del Castillo. "Sensory optimization of functional yogurts containing coffee byproducts as ingredients". Fourth International Conference on Cocoa Coffee and Tea. 25th - 28th June 2017. Centro Congressi Torino Incontra, Turin, Italy. Poster.
18. M. Iriondo-DeHond, A**. Iriondo-DeHond**, B. Fernández-Gómez, E. Miguel, M. D. del Castillo. "Optimización de la formulación y la calidad sensorial de nuevos lácteos funcionales". Jornadas Científicas CIAL Fórum. 16th - 17th November 2016. Research Institute of Food Science (CIAL, UAM-CSIC), Madrid, Spain. Poster.
19. **A. Iriondo-DeHond**, A. Haza, A. Avalos, M.D. del Castillo, P. Morales. "Protective effects of coffee silverskin extract against benzo(a)pyrene induced DNA damage". 20th International Conference of FFC - 8th International Symposium of ASFFBC: "Functional and Medical Foods for Chronic Diseases: Bioactive Compounds and Biomarkers". 22nd -23th September 2016. The Joseph B. Martin Conference Center at Harvard Medical School, Boston, USA. Poster.
20. **A. Iriondo-DeHond**, A. Haza, A. Avalos, M.D. del Castillo, P. Morales. "Evaluacion del efecto citotóxico y genotóxico del extracto de cascarilla de café en células HepG2". 2nd Congreso Iberoamericano de Ingenieria de los Alimentos. 13th - 14th November 2016. Punta del Este, Uruguay. Poster.
21. **A. Iriondo-DeHond**, A. Molina, M.D. del Castillo. "Potencial dermacéutico de extractos de café". 7th International Symposium of Food Development and Innovation: "Challenges, progress and innovation in food processing", 7th - 9th October 2015. LATU, Montevideo, Uruguay. Poster.
22. B. Fernández, C. Prieto, C. Marín, A. Revilla, A., **A. Iriondo**, C. González, F. González, I. Liste. "Generation of induced pluripotent stem cells (iPSCs) using non-viral vectors". ESGCT and SETGyC Collaborative Congress. European Society of Gene and Cell Therapy and Sociedad Española de Terapia Génica y Celular. 25th -28th October 2013. Madrid. Poster.

International programs

Participation in the From Waste to Worth program by EIT FOOD (March – December 2019). The program consisted in the development of a food by-product database to contribute to the sustainability of the food industry. A. Iriondo-DeHond

worked together with other students from Spain to develop the database. The following activities were performed as part of the program:

- One research stay in the University of Hohenheim, Germany, to work on the database development.
- Seven-month involvement in the database development, collecting data from the interested companies.

Supervision of undergraduate research

2019 – Co-supervisor of the internship of Noah Bader-Fourney, "Development of healthy novel beverages from coffee cascara", CSIC-OLESAY, University of Puget Sound, Washington, USA.

2019 – Co-supervisor of the internship of Jennifer Ronderos, "Development of healthy novel beverages from coffee cascara", CSIC-OLESAY, University of Florida, Florida, USA.

2019 – Co-supervisor of the internship of Lucía Huertas Díaz, "Efecto de los compuestos bioactivos de subproductos del café en la nefrotoxicidad inducida por AGEs", Degree in Bioquímica, Universidad Autónoma de Madrid, Spain.

2019 – Co-supervisor of the final degree project of Marta López Parra, "Estudio *in vitro* del efecto de la malabsorción de fructosa en la absorción de triptófano", Degree in Ciencias de la Alimentación, Universidad Autónoma de Madrid, Spain.

2019 – Co-supervisor of the final degree project of Alina Dubodel, "Consumo de azúcares añadidos y actividad física en adolescentes escolarizados en el municipio de Tres Cantos", Degree in Nutrición Humana y Dietética, Universidad Autónoma de Madrid, Spain.

2019 – Co-supervisor of the Master's Thesis of Sonia Baeza Labrador, "Bioaccesibilidad de los compuestos antioxidantes y antiinflamatorios de la cáscara de café y los ingredientes derivados", Master in Innovación y Desarrollo de Alimentos de Calidad, Universidad de Castilla-La Mancha, Spain.

2019 – Co-supervisor of the Master's Thesis of Ana Sofía Elizondo, "Desarrollo y caracterización de una bebida instantánea, antioxidante y sostenible a base de extracto de cáscara de café", Master in Nuevos Alimentos, Universidad Autónoma de Madrid, Spain.

2019 – Co-supervisor of the internship of Sonia Baeza Labrador, "Valorización de la cascarilla de café y de su fracción de alto peso molecular rica en melanoidinas", Master in Innovación y Desarrollo de Alimentos de Calidad, Universidad de Castilla-La Mancha, Spain.

2019 – Co-supervisor of the internship of Álvaro Gayo Medina, "Estudio de la tolerancia gastrointestinal de yogures ricos en fibra", Degree in Nutrición Humana y Dietética, Universidad Autónoma de Madrid, Spain.

2018 – Co-supervisor of the final degree project of Andrea Talasac Guerrero, "Efecto de compuestos fitoquímicos de café verde en la actividad de la alfa amilasa intestinal", Degree in Ciencias de la Alimentación, Universidad Autónoma de Madrid, Spain.

2018 – Co-supervisor of the final degree project of Paula Belotto Fernández, "Evaluación de la formación de productos de la reacción de Maillard derivados del triptófano simulando condiciones del lumen intestinal", Degree in Ciencias de la Alimentación, Universidad Autónoma de Madrid, Spain.

Training courses

1. Course: "Ethics and social responsibility of research" given by the DEMOSPAZ Institute, from February 22nd to March 22nd, 2018 (10 hours).
2. Course: "Advanced Methodology in Cellular Physiology" given by the University of Extremadura, 5 weeks, October of 2017 (on-line).
3. Course: Obtaining Function B (Euthanasia of Animals) and Function C (Realization of Procedures) of animal experimentation. October, 2016. Recognized by the General Directorate of Agriculture and Livestock of the Community of Madrid on April 4, 2016 (Reference 10 / 069861.9 / 16 dated 08/04/2016).
4. Course: Realization of 140 hours of practices to obtain the Training of the Community of Madrid to handle animals used, bred or supplied for experimental purposes and other scientific purposes, including teaching; in the Molecular Biology Center (CBMSO) supervised by Dr. Mª Dolores del Castillo in the framework of the authorized project with PROEX number 011/17.
5. Course: "Calibration and verification of automatic pipettes". May 2015. CIAL (UAM-CSIC).
6. Seminar: "I Jornada para Doctorandos del CSIC", Consejo Superior de Investigaciones Científicas. Madrid, Spain. 21st June, 2019.
7. Seminar: "Communication and Scientific Dissemination", Universidad Autónoma de Madrid. 15th January, 2019.

8. Seminar: "Jornada de iniciación a carreras profesionales para investigadores más allá del mundo académico". Departamento de Posgrado del CSIC. 14th November, 2018.
9. Seminar: "Día Mundial del Pan", Pan Cada Día, Hotel Miguel Ángel, Madrid. 16th October, 2018.
10. Seminar: "Introducción al diseño experimental para experimentos con animales de laboratorio" by Dr. Carlos Oscar Sánchez Sorzano, in Centro de Biología Molecular Severo Ochoa (CBMSO), Madrid, 31st May 2017 (2 hours).
11. Seminar: "Consumo Sostenible de Café y Salud", Townhall of Alcobendas, Madrid, 5th May, 2016.
12. Seminar: "Jornada formativa sobre Política de la Unión Europea en contaminantes de piensos y alimentos", Colegio Oficial de Veterinarios de Madrid, 20th of April, 2016.

Scientific transfer activities

1. Participation in "Noche de los Investigadores" at Medialab Prado in Museo del Prado de Madrid. 27th of September 2019. Madrid, Spain.
2. Teaching seminar "Bioaccesibility, metabolism, and excretion of lipids composing spent coffee grounds" at the Instituto de Investigación en Ciencias de la Alimentación (CIAL, UAM-CSIC). 13th of September 2019. Madrid, Spain.
3. Teaching workshop "Descubriendo los beneficios para la salud de nuevas bebidas de café" for students from Campus Científicos de Verano of FECYT in the Annual Food Agenda Project of EIT Food. 15th and 22nd July 2019. Madrid, Spain.
4. Participation in the "Campamento Urbano del Museo Nacional de Ciencias Naturales" in the Annual Food Agenda Project of the EIT Food Program. 11th and 25th of July 2019. Madrid, Spain. (http://annualfoodagenda.com/event-item/campamento-urbano-museo-de-verano/)
5. Participation in the Pre-Contest Phase of "Three Minute Thesis" 2019, at the Universidad Autónoma de Madrid, Spain. May 30th, 2019
6. Participation in Feria de Madrid por la Ciencia e Innovación 2019 in the Annual Food Agenda of the EIT Food Program. Madrid, Spain. 30th of March 2019.
7. Participation in the "Día Internacional de la Mujer y la Niña en la Ciencia" with the activity of "Descubriendo los beneficios para la salud de nuevas bebidas de café" at the Instituto Rosa Chacel to students of the International Baccalaureate, Madrid, Spain. 15th February 2019. (https://www.cial.uam-csic.es/3359/biociencia-de-los-alimentos-celebra-el-dia-de-la-mujer-y-la-nina-en-el-ciencia/)

8. Participation in the "Día Internacional de la Mujer y la Niña en la Ciencia" with the activity of "Descubriendo los beneficios para la salud de nuevas bebidas de café" at the Centro de Innovación Gastronómica, Madrid, Spain. 8th February 2019. (https://www.cial.uam-csic.es/3359/biociencia-de-los-alimentos-celebra-el-dia-de-la-mujer-y-la-nina-en-el-ciencia/)
9. Participation in "Semana de la Ciencia" with the activity of "¿Contienen azúcares las bebidas que más consumimos?". 6th and 7th November 2018. CIAL (UAM-CSIC), Madrid, Spain.
10. Organization and speaker in the Summer Course "Descubre el Queso: Denominaciones de Origen y Análisis Sensorial" of the Universidad Rey Juan Carlos, Madrid, Spain. 29th June 2018. (https://uverano.urjc.es/20717/detail/cv-j-deq.-descubre-el-queso-denominaciones-de-origen-y-analisis-sensorial.html)
11. Participation in the "Día Internacional de la Mujer y la Niña en la Ciencia" presenting our research group in the Instituto Pintor Antonio López from Tres Cantos, Madrid, Spain (students from 2º ESO). 7th February 2018.
12. Participation in the "Día Internacional de la Mujer y la Niña en la Ciencia" with the activity of "¿Contienen azúcares las bebidas que más consumimos?" in the Colegio Zola from Villafranca del Castillo, Madrid, Spain (students from 4º Primaria). 26th January 2018.
13. Participation in "Día Internacional de la Seguridad Alimentaria" organized by ALCyTA giving the workshop of "Aditivos Alimentarios: Combatiendo la Quimiofobia y el Desconocimiento", at Universidad Autónoma de Madrid, Sapin. 17th November 2017.
14. Participation in "Semana de la Ciencia" with the activity of "¿Contienen azúcares las bebidas que más consumimos?". 14th and 15th November 2017. CIAL (UAM-CSIC). Madrid, Spain.
15. Participation in TV show "Devorando mitos", in Comando Actualidad TVE. 1st of March 2017. Madrid, Spain.
16. Participation in "Semana de la Ciencia" with the activity of "¿Contienen azúcares las bebidas que más consumimos?". November 2015. CIAL (UAM-CSIC). Madrid, Spain.

Social entrepreneurship

(2017- present) Co-founder and secretary of the non-profit association "AlimentUS", aiming to transfer scientific knowledge in Food Science and Nutrition to the general public. The association was founded by students from the Bioscience research group (CIAL-CSIC) and IMIDRA.

“If I could tell you what it meant, there would be no point in dancing it”

Isadora Duncan